From THE DEPARTMENT OF MOLECULAR MEDICINE
AND SURGERY
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NEW MOLECULAR TOOLS FOR PRENATAL DIAGNOSIS

Ellika Sahlin

Karolinska Institutet

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NEW MOLECULAR TOOLS FOR PRENATAL DIAGNOSIS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Ellika Sahlin

Principal Supervisor:
Associate professor Erik Iwarsson
Karolinska Institutet
Department of Molecular Medicine and Surgery

Co-supervisors:
Professor Magnus Nordenskjöld
Karolinska Institutet
Department of Molecular Medicine and Surgery

Dr Agne Liedén
Karolinska Institutet
Department of Molecular Medicine and Surgery

Dr Peter Gustavsson
Karolinska Institutet
Department of Molecular Medicine and Surgery

Professor Sten Linnarsson
Karolinska Institutet
Department of Medical Biochemistry and Biophysics

Opponent:
Professor Joris Vermeesch
Katholieke Universiteit Leuven
Department of Human Genetics

Examination Board:
Professor Mikael Norman
Karolinska Institutet
Department of Clinical Science, Intervention and Technology

Professor Marie-Louise Bondeson
Uppsala University
Department of Immunology, Genetics and Pathology

Professor Ove Axelsson
Uppsala University
Department of Women's and Children's Health

Skäms inte för att du är människa, var stolt!
Inne i dig öppnar sig valv bakom valv oändligt.
Du blir aldrig färdig, och det är som det skall.

Tomas Tranströmer

ABSTRACT

Prenatal diagnosis enables identification of severe disease in the fetus, and allows for planning and management of future pregnancies if an underlying genetic mechanism is identified. The studies included in this thesis have taken advantage of the dramatic progress in medical genetics, in order to develop and evaluate new procedures to diagnose genetic disorders in fetal life. A correct diagnosis is important not only for the management and counseling of the patient or couple, but may also have an impact on the extended family, as there may be undetected carriers.

The incidence of stillbirth in Sweden, *i.e.* fetal death occurring at gestational week ≥22, has essentially remained constant since the 1980's, and despite thorough investigation, many cases remain unexplained. In **Paper I,** clinical chromosome analysis results from 481 stillbirth cases were compiled, and a subgroup of 90 cases were analyzed using chromosomal microarray (CMA) to study the potential benefits of the method. The conventional analysis detected chromosomal aberrations in 7.5% of the cases. CMA additionally identified two known syndromes, one disruption of a known disease gene, and 26 variants of unknown significance. Furthermore, CMA had a significantly higher success rate than cytogenetic analysis (100% vs. 80%, $p<0.001$). As CMA increased both the success rate and diagnosis rate, we concluded that it is a valuable tool in stillbirth investigation. In **Paper II,** DNA from 290 stillbirth cases was analyzed by massive parallel sequencing of 79 genes associated with heart disease. Sixty-two cases (21.4%) had putative pathogenic variants in genes associated with channelopathies (53%), cardiomyopathies (24%) or congenital heart defects (23%). Screening for pathogenic variants in genes associated with heart disease might be a valuable complement in cases where the conventional investigation does not reveal the underlying cause of fetal demise.

Fetal tissue calcifications are occasionally noted during autopsy or ultrasound, but their biological importance is largely unexplored. In **Paper III,** a case-control study including 151 calcification cases and 302 matched controls was performed, to identify factors associated with calcifications. Chromosomal abnormalities, detected by conventional chromosome analysis or quantitative fluorescence-polymerase chain reaction, were significantly more common in cases compared with controls; 50% vs. 20% ($p<0.001$). When comparing cases and controls with chromosomal abnormalities, the cases had a significantly higher prevalence of malformations (96% vs. 77%, $p=0.002$). We concluded that fetal tissue calcifications are associated with chromosomal abnormalities, especially in combination with malformations.

Non-invasive prenatal testing (NIPT) for fetal aneuploidy, based on sequencing analysis of cell-free fetal DNA in maternal plasma, has recently been made possible. Current methods require DNA amplification prior to sequencing, which inevitably leads to a bias because some DNA fragments amplify more efficiently than others. In **Paper IV,** an amplification-free NIPT protocol was evaluated on 31 samples, of which 15 had confirmed aneuploidies. All aneuploidies were correctly classified, except in one case where cytogenetic testing had confirmed the presence of both trisomy 18 and XXY, but NIPT failed to identify the extra X chromosome. Further analyses revealed that the fetus was mosaic, which probably explains why the extra X chromosome was missed. We concluded that the amplification-free protocol can potentially be used for NIPT, as it could distinguish aberrant samples from healthy controls. Compared to other NIPT protocols, it can be used on smaller quantities of input DNA, reduces GC bias and increases genome coverage.

In **Paper V,** we aimed to gain an increased understanding of pregnant women's awareness, attitudes, and preferences concerning prenatal testing with emphasis on NIPT, before its introduction into Swedish healthcare. In total, 1,003 pregnant women were recruited to fill in a questionnaire at nine maternity clinics located in different areas of Stockholm. The overwhelming majority of the women (91%) considered examinations aiming to detect fetal abnormalities to be good. Regarding NIPT, 60% stated that they had heard about the method previously, yet 74% would like to use the test if available. The main factor affecting the women's decision to undergo prenatal chromosomal screening was worry about the baby's health (83%), followed by the desire to have as much information as possible about their fetuses (55%).

The results from the studies have provided support for the value of various genetic analysis methods in the field of prenatal diagnosis, as well as insights to the prevalence of genetic aberrations in fetuses.

LIST OF SCIENTIFIC PAPERS

The thesis is based on the following articles:

I. **Sahlin E**, Gustavsson P, Liedén A, Papadogiannakis N, Bjäreborn L, Pettersson K, Nordenskjöld M, Iwarsson E. *Molecular and cytogenetic analysis in stillbirth: results from 481 consecutive cases.* Fetal diagnosis and therapy. 2014;36(4):326-32.

II. **Sahlin E,** Gréen A, Gustavsson P, Liedén A, Nordenskjöld M, Papadogiannakis N, Pettersson K, Nilsson D, Jonasson J, Iwarsson E. *Identification of pathogenic single nucleotide variants (SNVs) associated with heart disease in 290 cases of stillbirth.* Manuscript.

III. **Sahlin E**, Sirotkina M, Marnerides A, Iwarsson E & Papadogiannakis N. *Fetal calcifications are associated with chromosomal abnormalities.* PloS One. 2015;10(4):e0123343.

IV. Karlsson K, **Sahlin E**, Iwarsson E, Westgren M, Nordenskjöld M, Linnarsson S. *Amplification-free sequencing of cell-free DNA for prenatal non-invasive diagnosis of chromosomal aberrations.* Genomics. 2015 Mar;105(3):150-8.

V. **Sahlin E**, Nordenskjöld M, Gustavsson P, Wincent J, Georgsson S & Iwarsson E. *Positive attitudes towards non-invasive prenatal testing (NIPT) in a Swedish cohort of 1,003 pregnant women.* PLoS One. 2016 May 19;11(5):e0156088.

CONTENTS

ABBREVIATIONS

AFR	Amplification-free read
Array-CGH	Array-based comparative genomic hybridization
bp	Basepairs
BrS	Brugada syndrome
CADD	Combined annotation dependent depletion
CI	Confidence interval
cfDNA	Cell-free DNA
cffDNA	Cell-free fetal DNA
CHD	Congenital heart defect
CMA	Chromosomal microarray
CNV	Copy number variant
CPM	Confined placental mosaicism
CPVT	Catecholaminergic polymorphic ventricular tachycardia
CVS	Chorionic villus sampling
DNA	Deoxyribonucleic acid
ExAC	Exome Aggregation Consortium
FCT	First trimester combined test
FoSTeS	Fork-stalling and template switching
HCM	Hypertrophic cardiomyopathy
kb	Kilobases, thousands of basepairs
LoF	Loss-of-function
LQTS	Long QT syndrome
Mb	Megabases, millions of basepairs
MMBIR	Micro-homology mediated break induced repair
MPS	Massive parallel sequencing
NAHR	Non-allelic homologous recombination
NHEJ	Non-homologous end-joining
NGS	Next-generation sequencing
NIPT	Non-invasive prenatal testing
NRC	Normalized read count
PCR	Polymerase chain reaction
PPV	Positive predictive value
QF-PCR	Quantitative fluorescence-polymerase chain reaction
RhD	Rhesus D
SIDS	Sudden infant death syndrome
SNP	Single nucleotide polymorphism
SNV	Singe nucleotide variant
STR	Short tandem repeat
VOUS	Variant of unknown significance
WES	Whole exome sequencing
WGS	Whole genome sequencing

1 INTRODUCTION

Prenatal diagnosis enables detection of severe disease already present in the fetus. Information about the fetal health status is important for pregnancy management, for deciding whether to continue the pregnancy, and for the planning and management of possible complications associated with the delivery and caretaking of the child after birth. Additionally, in case of a genetic disorder, identification of the underlying genetic mechanism enables estimations of the recurrence risk in future pregnancies. This project has taken advantage of the dramatic progress in medical genetics, in order to develop and evaluate new procedures to diagnose genetic disorders in fetal life. A correct diagnosis is important not only for the management and counseling of the patient or couple, but in the case of a genetic disease it may also have an impact on the extended family, as there may be undetected carriers also at risk of having affected offspring.

1.1 EMBRYONIC AND FETAL DEVELOPMENT

The human gestational period lasts for roughly 40 weeks, and is divided into three trimesters; each comprising three months. During this time a single cell, the zygote, will develop into a full-grown fetus. The gestational weeks are counted based on the first day of the last menstrual period, with fertilization taking place in the end of the second gestational week. The first eight weeks following fertilization are known as the embryonic period, which includes several developmental processes. The first embryonic cell divisions are called cleavages, as they result in no increase in the embryonal size. Cleavages occur until the embryo consists of a compact ball of equal cells; the morula. As the cells continue dividing, the compact morula will eventually develop into a hollow blastocyst. The blastocyst is composed of two distinct cell types; the embryoblasts, which constitute the inner cell mass, and the trophoblasts surrounding them. The inner cell mass will eventually develop into the fetus, whereas the trophoblasts will become the placenta. In the process of gastrulation, which occurs during the third embryonal week, the inner cell mass will evolve into three distinct germ layers; the ectoderm, the mesoderm and the endoderm. This is a crucial event as the layers are the first step towards organogenesis, *i.e.* formation of precursors to all organs in the body. Organogenesis lasts throughout the embryonic period, *i.e.* until gestational week 10.

The fetal period, which begins in gestational week 11, is mainly devoted to the maturation of all organs, and fetal growth. By the time at which the fetal period is about to begin, the embryo is approximately 1 cm in size, and weighs around 8 grams. By term, the fetus will be 50 cm in length and weigh around 3 kg. Most of the growth in length takes place during the second trimester, whereas most of the weight is put on in the third trimester[1].

1.1.1 Stillbirth

Pregnancy loss is very common; only around half of conceptuses (*i.e.* products of conception) will make it until term[2]. However, most pregnancy losses occur in early pregnancy. Stillbirth, on the other hand, is defined as ante- or intrapartal death occurring from 22 completed gestational weeks. It has an incidence of 3-4/1,000 live births in Sweden, according to the Swedish Medical Birth Register (data from 2014)[3], and can be caused by several factors, such as genetic mechanisms, maternal conditions, infections, placental insufficiency and placental abruption[4,5]. The proportion of cases that is due to genetic causes is still uncertain and depends on how the cases are categorized. In many studies, all malformations are considered to have a genetic background, and the stillbirths are hence classified as such, even if the underlying genetic cause is not identified. Using traditional chromosome analysis, 6-17% of the stillborn has a proven chromosomal abnormality[6].

All cases of stillbirth in the Stockholm County pass a thorough investigation, with the aim of identifying the underlying cause of fetal demise. The investigation includes physical examination and autopsy, infectious disease testing and chromosome analysis. Yet, after a full investigation, up to 20% are classified as unexplained[7]. The incidence of stillbirth in Sweden has essentially remained constant since the 1980's[3], and better knowledge of the underlying mechanisms is needed in order to achieve a reduction in the stillbirth rate. The development of chromosome analysis methods with a higher resolution than the traditional methods has drawn attention towards the clinical impact of submicroscopic chromosomal abnormalities, which previously have remained undetected. Additionally, studies suggest that a proportion of stillbirth cases are due to heart disease, mainly channelopathies, *i.e.* defects in cardiac ion channels leading to a disrupted action potential propagation and thereby causing development of arrhythmias[8]. It is of importance to determine the underlying cause, as a history of stillbirth is associated with an increased recurrence risk in future pregnancies[9,10]. Furthermore, better understanding of the reason why the pregnancy ended in a stillbirth can help the parents deal with the loss.

1.1.2 Fetal tissue calcifications

During fetal autopsy, and more rarely also at ultrasound analysis, fetal tissue calcifications are occasionally noted, mainly in the liver. Most of them are so-called microcalcifications that can only be visualized using microscopy, but if sufficiently large, they can be detected with the naked eye. As opposed to many other morphological features, which in many cases are damaged in the autolysis process, fetal liver calcifications can be visualized on histological sections regardless of the degree of autolysis[11]. Although the occurrence of calcifications has been known for a long time, their biological importance is largely unexplored. Previous studies have indicated associations with infection[12-14], circulatory compromise[11,15], malformations or chromosomal abnormalities[11,12,16-18].

1.2 EARLY FORMS OF PRENATAL DIAGNOSIS

The development of the unborn child has fascinated people for many centuries. The oldest known documented representation of a human embryo dates back to 3,000-2,500 BC and pictures embryos at approximately 9 gestational weeks, discovered in a temple dedicated to a fertility goddess in Malta[19]. However, it would take another 5,000 years until a fetus could be visualized *in utero.* In 1923, the benefit of using x-rays to study fetuses was presented. Using this method, fetal position, bone structure and gestational age could be assessed[20], but over the next decades it became increasingly evident that x-rays had harmful effects on the fetus. By 1975, strong evidence showed that x-rays could lead to miscarriage, fetal malformation, increased risk for childhood cancer and changes in the sex ratio[21,22]. During the same period of time, ultrasound imaging emerged as an alternative to x-ray, and quickly became the method of choice[23], as it still is today. A beam of ultrasound is used for scanning, and a computer analyzes the pattern of the returning echoes. Because tissues of different densities reflect sound differently, bodily structures of the fetus can be visualized.

In parallel with the development of fetal visualization techniques, methods for fetal genetic testing began to evolve. The first step towards prenatal genetic diagnosis was made by the Canadians Barr and Bertram in 1948. They discovered that cells derived from females of many species, including humans, contained a condensed body in the nuclear periphery[24]. They hypothesized that this body, which they called the sex chromatin but subsequently became known as the Barr body, could be used for sex determination if a sufficient amount of cells were studied. Methods to collect fetal cells were already available as amniocentesis, *i.e.* withdrawal of amniotic fluid, had been developed to treat fluid excess (polyhydramnios)[25]. By combining these two techniques, the fetal sex could be determined with high accuracy, which was valuable information in cases where a sex-linked disease, such as hemophilia, was present in a family[26]. Approximately ten years after the discovery of the Barr body, the number of human chromosomes was established, and shortly thereafter it was discovered that some congenital syndromes, *e.g.* Down syndrome and Klinefelter syndrome, are due to an abnormal number of chromosomes[27]. In the early 1970's, a technical innovation – chromosome banding – took chromosome analysis to a new level. It enabled identification of specific chromosomes, as well as structural chromosomal changes[28].

1.3 GENETIC ABERRATIONS

The human genome is almost two meters in length. To fit the genome in each cell nucleus, which in a typical cell is 6µm in diameter, it is tightly packed into chromosomes. Each chromosome contains a single molecule of deoxyribonucleic acid (DNA), which is packed in multiple levels of condensation by histones and other proteins (Figure 1).

The DNA itself is built by nucleotides, which consists of three components; a phosphate group, a deoxyribose sugar group, and a nitrogen base. The phosphate and sugar together form the backbone of the DNA molecule, whereas there are four different types of bases;

adenine (A), thymine (T), cytosine (C), and guanine (G). All genetic information is contained in the sequence of the bases. The DNA is a double-stranded molecule, *i.e.* it consists of two strands of nucleotides that are held together by hydrogen bonding between the bases (A with T or C with G). The complementary structure of DNA ensures reliable replication of the genome, which thus can be transmitted to the next generation. However, errors do occur, and can range from a change of a single nucleotide to gain or loss of whole chromosomes. Hence, genetic aberrations can result in a wide variety of phenotypes, and each type of aberration requires its own special set of techniques to identify them.

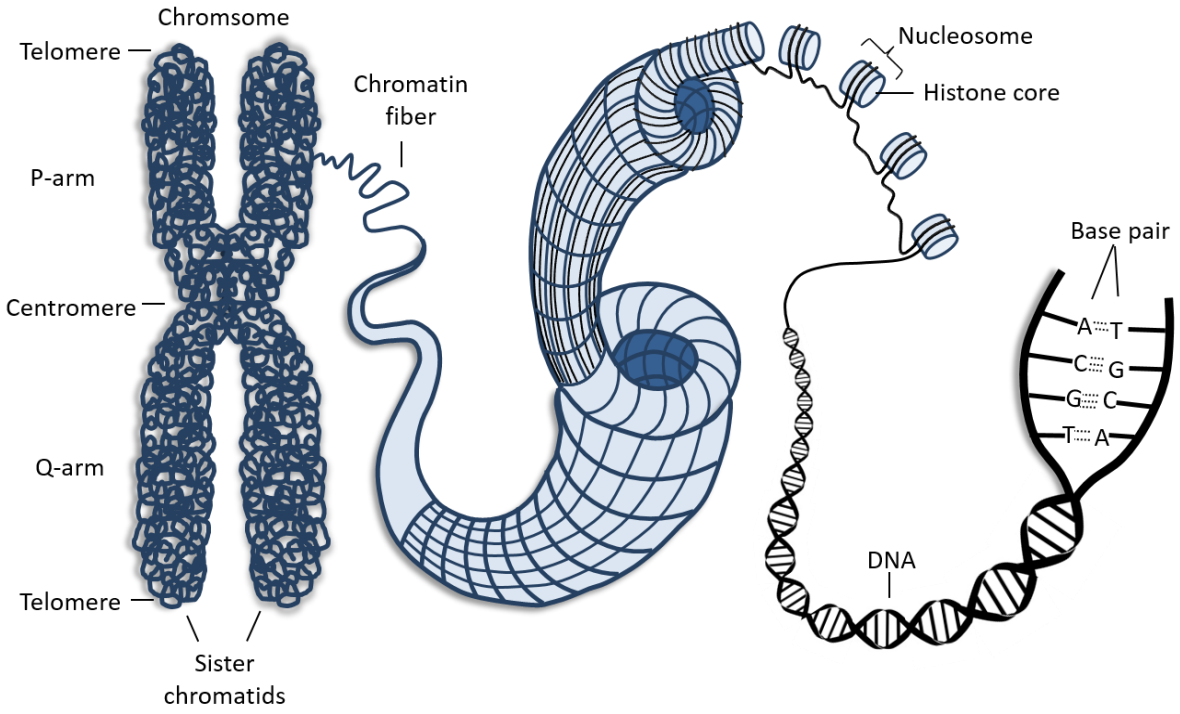


Figure 1. The hierarchical structure of DNA packed in a chromosome.

1.3.1 Numerical chromosomal abnormalities

The haploid human genome contains 23 chromosomes. The haploid chromosome set is found in the gametes, *i.e.* oocytes (“eggs”) or sperm cells, which are produced by meiotic cell division. Following fertilization, one oocyte and one sperm together form a zygote, *i.e.* the first cell of a new individual. The zygote is consequently diploid; it contains 46 chromosomes. By mitotic cell division, the zygote will transmit the diploid chromosome set to its daughter cells. The 46 chromosomes include 22 pairs of autosomes, named 1-22, and one pair of sex chromosomes, which is XX in females or XY in males. One chromosome in each pair derives from the oocyte whereas the other derives from the sperm cell. The total number of chromosomes as well as the sex chromosomes in diploid cells is referred to as the karyotype of an individual. A normal karyotype in a female and a male is hence 46,XX and 46,XY, respectively.

As indicated by its name, a numerical chromosomal abnormality refers to an abnormality where the number of chromosomes differs from the normal 46. There are two main types of numerical abnormalities; aneuploidy and polyploidy. Aneuploidy is a phenomenon which results from gain or loss of single whole chromosomes. A gain of a chromosome will lead to that there are three homologs instead of two in each cell, *i.e.* a trisomy. Conversely, loss of a chromosome results in a monosomy. Aneuploidies usually originate from an error known as non-disjunction occurring in the meiotic cell division, or during mitosis in the early embryo. The most common aneuploidy in early embryos and fetuses involve chromosome 16, followed by the acrocentric autosomes (which are chromosomes 13, 14, 15, 21, and 22)[29,30]. However, most of these will not result in a viable offspring. The most common aneuploidies observed in the late fetus (≤20 weeks) as well as in born children are trisomy 21 (Down syndrome), trisomy 18 (Edward syndrome), trisomy 13 (Patau syndrome) as well as aneuploidies involving the sex chromosomes[31].

A polyploidy infers that one or more complete sets of chromosomes are gained in a cell. Gain of one extra set results in a triploidy, *i.e.* 69 chromosomes, and a tetraploidy consequently means that there are 92 chromosomes in each cell. The formation of polyploidy can occur by fertilization of the oocyte by multiple sperm, or that the oocyte contained a diploid set of chromosomes prior to fertilization[32]. Polyploidy is usually not compatible with life in human, but is not uncommon in the early embryo or fetus, occurring in 1-3% of conceptuses[32,33]. In addition to aneuploidy and polyploidy there is mixoploidy, which is a state in where multiple cell lines, which can differ in regard to their chromosomal setup, are present in the same individual. If the cell lines are derived from the same zygote, it is known as mosaicism, whereas if they are derived from different zygotes it is known as chimerism.

In the context of prenatal diagnosis, numerical chromosomal abnormalities constitute the group of aberrations that has traditionally been in focus. The risk of numerical aberrations increases with maternal age, although the mechanism behind this increase is not fully understood.

1.3.2 Structural chromosomal abnormalities

Structural chromosomal abnormalities are, as the name implies, changes in the chromosomal structure. There are different types of structural variants, including translocations, inversions, deletions and duplications, as well as complex rearrangements. Translocations commonly occur when two or more chromosomes break and the loose ends are joined together in the wrong order, but they can also arise from mechanisms associated with replication. A special type is the Robertsonian translocation, which is caused by fusion of the long arms of two acrocentric chromosomes. Translocations can be balanced, meaning that although in a rearranged order, all genetic information is present in the correct amount. Unless the breakpoints are located in, or in close proximity to, a disease causing gene, balanced translocations usually do not give rise to a clinical phenotype in the carrier, but can cause problems in meiosis resulting in unbalanced gametes. As a result, balanced translocations are

a well-known cause of infertility and repeated miscarriages[34,35], but can also result in offspring with developmental delay and/or congenital malformations due to the fact that the translocations can be inherited in an unbalanced manner. An inversion, in contrast to a translocation, involves only one chromosome. The common mechanism of formation is chromosome breakage at two sites, whereupon the middle part is rotated 180° before reintegration into the same chromosome. As with translocations, an inversion usually does not cause symptoms in the carrier, but can give rise to unbalanced gametes.

Deletions and duplications, *i.e.* loss or gain of chromosome segments, are also known as copy number variants (CNVs). CNVs can arise from several mechanisms, such as non-allelic homologous recombination (NAHR), non-homologous end joining (NHEJ), micro-homology mediated break induced repair (MMBIR)/Fork-stalling and template switching (FoSTeS)[36], or of inheritance of a translocation or inversion in an unbalanced manner. Depending on their location in the genome and the copy number status (loss or gain), CNVs can influence gene expression and phenotypic variation, including development of disease. However, a lot of CNVs are benign normal variants that are found in the general population.

Because of its limited resolution, traditional chromosome analysis only can only detect CNVs if they are very large, minimum 5-10 megabases (Mb, millions of basepairs). Therefore, until recently, the impact of smaller CNVs has been largely unknown in the context of fetal development. However, submicroscopic CNVs are today recognized as a major cause of genetic disorders postnatally, estimated to cause approximately 14-17% of intellectual disability and/or multiple congenital anomalies[37,38].

1.3.3 Nucleotide sequence variants

The smallest genetic aberrations are those affecting only one or a few nucleotides. The human genome has a built-in rate of mutation, *i.e.* alteration of the nucleotide sequence, due to the DNA polymerases which replicate the DNA in each cell cycle, have error rates ranging from approximately 1/10,000 to 1/100,000 basepairs (bp)[39]. This means that every individual will have some genetic variants that are not found in either of the parents, so-called *de novo* variants. From an evolutionary perspective, this is a good thing as it ensures genetic diversity in the population. However, depending on where the errors occur, they can have deleterious effects on an individual level. In its strict sense, the word mutation means a nucleotide change that is present in less than 1% of the population, and does not infer any information about its functional impact. However, the term is commonly used as a synonym to a pathogenic variant, which has become a source of confusion. In accordance with current guidelines[40], mutations will henceforth collectively be referred to as sequence variants, including single nucleotide variants (SNVs) and small insertions and deletions (indels). Consequently, a pathogenic mutation will be referred to as a pathogenic variant.

Out of the approximately 3 billion basepairs that constitute a haploid genome, 1.5% compose exons of protein-coding genes[41]. In these genes, nucleotide triplets, *i.e.* codons, create a code

which can be translated into a sequence of amino acids which are the building blocks of proteins. A variant in a protein-coding gene can for example give rise to a change in the amino acid sequence (missense variant), introduction of a stop codon (nonsense variant), or that the reading frame of the codons is shifted (frameshift variant). Disease caused by pathogenic variants in protein-coding genes can be either dominant, *i.e.* one altered allele is enough to develop disease, or recessive, *i.e.* both alleles must be altered for disease to occur. The functional impact of a previously unknown variant can be difficult to determine, and is also complicated by the fact that not everyone with a deleterious variant will develop disease (reduced penetrance), as well as that a disease can cause different symptoms with a varying severity in different individuals (variable expressivity).

In prenatal diagnosis, nucleotide sequence variants have traditionally only been analyzed if the parents are known disease carriers. As opposed to numerical variants, which are associated with maternal age, *de novo* SNVs are more commonly transmitted from males, as they will accumulate over time in spermatocytes[42], on average at a rate of two new SNVs per year[43].

1.4 PRENATAL DIAGNOSIS VS PRENATAL SCREENING

A definitive diagnosis of fetal chromosomal abnormalities during an ongoing pregnancy can currently only be achieved by analysis of invasively obtained fetal cells. The invasive procedures include amniocentesis, *i.e.* withdrawal of amniotic fluid, and chorionic villus sampling (CVS), *i.e.* a placental biopsy. CVS has the advantage that it can be performed earlier in pregnancy than amniocentesis (week 10-12 vs. week 15-16), but has the disadvantage that the placental karyotype in rare cases differs from the fetal, most often as confined placental mosaicism (CPM). As both amniocentesis and CVS are invasive methods, they are associated with some risks. Although recent data suggest that invasive testing is very safe[44,45], it has traditionally been burdened with a non-negligible risk of miscarriage, commonly stated as 1:200[46]. Additionally, the invasive procedure is often considered unpleasant for the pregnant woman, as well as it is expensive as it requires a fetal medicine expert to perform the test. Furthermore, the standard way of identifying chromosomal abnormalities in the fetal cells collected by invasive testing is by conventional karyotyping, which has some disadvantages. As mentioned previously, the resolution of the analysis is low, and the method is also hampered by long turn-around times because of the need for cell culture, which is not optimal regarding the time constraints in an ongoing pregnancy.

Taken together, prenatal diagnosis by invasive testing has never been either intended, nor suitable, for large-scale testing. Different screening methods have therefore evolved to identify pregnant women at high risk of fetal aneuploidy. As it is known that the aneuploidy risk increases with maternal age, an early recommendation was that only women of ≥35 years of age should be offered invasive testing. However, age alone is a poor criterion as it only identifies 25-30% of fetal aneuploidy[47]. In the 1990's, it was shown that the clear space of the

fetal neck, the nuchal translucency, was associated with chromosomal abnormalities, where higher values inferred a higher risk[48]. Maternal age together with the nuchal translucency measurement, was subsequently combined with concentration measurements of two pregnancy associated proteins in maternal blood; pregnancy associated plasma protein A (PAPP-A) and human chorionic gonadotropin (hCG). The combination of these parameters is known as the first trimester combined test (FCT) which today is a widely used screening test for fetal aneuploidy, with a detection rate of approximately 80%, and a false positive rate around 5%[46,47]. The outcome of FCT is a risk assessment of fetal aneuploidy. In Sweden, as well as in many other countries where FCT is used, "high risk" is defined as 1:200. In 2014, approximately 64% of the women in Stockholm County had an FCT, and 8% had an invasive test.

1.5 NON-INVASIVE PRENATAL TESTING (NIPT)

Prenatal diagnosis has recently entered a new era because non-invasive prenatal testing (NIPT) has become a clinical reality, rapidly spreading worldwide. In 1997, it was discovered that cell-free fetal DNA (cffDNA) is present in maternal blood during pregnancy[49]. This discovery was the first step towards NIPT. However it would take several years for the method to become successful because of major technical limitations, mainly due to the fact that the vast majority of the total cell-free DNA (cfDNA) in maternal plasma derives from the pregnant woman herself. In gestational week 10-20, the fetal DNA fraction is on average 10-15%, but can range from as low as 3% up to 30%[50,51]. The fetal fraction can be affected by several factors, such as maternal weight[52] and fetal aneuploidy[53,54].

The first widespread clinical use of NIPT was for two indications, *i.e.* sex determination[55] (in case of X-linked disease) and determination fetal Rhesus D (RhD) blood group status[56], which is important for correct management of RhD negative women carrying an RhD positive fetus. Common for these indications is that they both are based on paternally inherited alleles, and can therefore relatively easily be identified despite the maternal background. Detection of fetal aneuploidy puts higher demands on the method as it requires accurate counting of the number of chromosomes, rather than merely determining the presence or absence of a certain allele. However, fetal aneuploidy detection has recently been enabled by the technical improvement of massive parallel sequencing (MPS). For this task, different approaches have evolved, which are either based on whole-genome sequencing (WGS)[57-59], or different targeted strategies (targeting specific chromosomal regions[60] or single nucleotide polymorphisms, SNPs[61-63]). An extra chromosome in the fetus will lead to a higher amount of cffDNA derived from that particular chromosome, compared with reference chromosomes. By sequencing several million cfDNA fragments and thereafter counting how many fragments belong to each chromosome, the aneuploidy can be detected. Aberrant cfDNA profiles can however be caused by other factors; analysis with NIPT has for instance resulted in presymptomatic identification of cancers in pregnant women, by detection of chromosomal abnormalities derived from the tumor[64].

The detection rates of NIPT regarding fetal trisomies 21, 18 and 13 have been reported to 99.2%, 96.3% and 91.0%, respectively[65]. However, the positive predictive value (PPV), *i.e.* the probability that a positive result indeed is true, varies depending on the prevalence of the aneuploidy in the pregnant population being tested. For example, in an unselected pregnant population, the PPV for trisomy 21 is approximately 50-80%[66-68], and hence half of those singled out by NIPT may carry a normal fetus. NIPT is consequently not a diagnostic test, and a positive test result requires confirmation by invasive testing. However, as the accuracy of NIPT is much higher than current other screening methods, the majority of invasive tests could be avoided if NIPT was used as the primary screening.

Although referred to as fetal DNA, the cffDNA mainly derives from apoptotic trophoblasts and syncytiotrophoblasts in the placenta[69]. If future technical improvements will upgrade NIPT to a diagnostic test, its accuracy will therefore be similar to CVS.

1.6 ETHICAL CONSIDERATIONS

Prenatal diagnosis is a topic that engages many people, and the debate gained new life in response to the introduction of NIPT. Concerns have arisen that the test is "too simple" as it only requires a simple blood sample from the pregnant woman, and that it therefore could be considered a routine sample among others that many would have without sufficient reflection[70,71], or even that the pressure to have the test might increase as women might feel less justified to decline such a simple and risk free test[70]. Other concerns include the risk of trivialization of selective abortions[72], and that people with disabilities might face an increased stigmatization in the society[73]. As it has been shown that the complete fetal genome is represented in maternal plasma[74], there are concerns for a "slippery slope" effect, *i.e.* that the spectrum of traits being tested for will widen, and even include non-medical indications[75]. On the advantageous side, NIPT enables earlier testing, which allows for more time to prepare to care for a child with a syndrome, or for earlier pregnancy termination, depending on the decision by the parents. Additionally, as stated earlier, primary screening by NIPT drastically reduces the need for invasive procedures.

According to Swedish law, all pregnant women should be offered general information about prenatal diagnosis, and women at increased risk of having a child with a genetic disease should be offered additional information regarding genetic prenatal testing. Thereafter, it is up to the pregnant woman herself if she wants to perform any tests. It is highly important that expectant parents are provided sufficient information to be able to make carefully prepared choices, regardless if the choice is to do or not to do prenatal testing.

2 AIMS OF THE THESIS

The overall aim of this thesis was to develop and evaluate new procedures to diagnose genetic disorders in fetal life, and thereby improve prenatal care and increase our knowledge of the normal and abnormal fetal development.

The specific aims were:

1) to identify mechanisms causing stillbirth, anatomic abnormalities and tissue calcifications using different DNA-based methods (Paper I-III)

2) to evaluate NIPT using massive parallel DNA sequencing for the analysis of fetal aneuploidy; both technically (Paper IV) as well as to evaluate the attitude towards the method by pregnant women (Paper V)

3 MATERIALS AND METHODS

3.1 STUDY SUBJECTS

Due to clinical chromosome analysis which is part of the routine investigation, tissue samples from all stillbirth cases in Stockholm have been stored at the Department of Clinical Genetics as part of Biobank Karolinska, resulting in a unique material from consecutive stillbirth cases during several years. In **Paper I**, clinical results from chromosome analysis were compiled for 481 stillbirths, *i.e.* all cases that occurred in the Stockholm County during five years; 2008-2012. A subgroup of 90 cases, corresponding to all cases without a genetic diagnosis analyzed in 2010, was additionally analyzed by chromosomal microarray (CMA), to study the possible benefits of this method in the analysis of the etiology of stillbirth. In **Paper II**, 290 stillbirth cases without a previous genetic diagnosis were analyzed using a gene panel including 79 genes associated with various heart conditions and heart development, in order to increase our knowledge of the potential role of heart disease in stillbirth.

In **Paper III**, 151 fetuses with tissue calcifications were retrospectively identified from the archives of the Center for Perinatal Pathology at the Department of Pathology, Karolinska University Hospital, corresponding to all cases with registered fetal calcifications during 2003-2012. For each case, two controls were identified from the same archives. The controls were matched for gestational age and type of death (spontaneous or missed abortion, stillbirth, induced termination of pregnancy), in order to allow for a matched case-control study with the aim of identifying factors associated with fetal calcifications.

In **Paper IV**, plasma samples from 31 pregnant women were analyzed using an amplification-free protocol for non-invasive prenatal testing (NIPT), to evaluate its ability to detect fetal aneuploidies. Fifteen of the samples were donated from women whose fetuses had confirmed aneuploidies, whereas 16 were healthy controls.

Paper V included 1,003 pregnant women recruited at nine maternity clinics located in different areas of Stockholm. The women filled in a questionnaire covering awareness, attitudes, preferences for risk information and decision-making concerning prenatal examinations with emphasis on NIPT, before its introduction into Swedish healthcare.

3.2 CYTOGENETIC ANALYSIS AND QF-PCR

Traditional cytogenetic analysis by karyotyping is enabled by culturing cells and chemically arresting the cell cycle in metaphase. During this stage, the chromosomes are highly condensed, which allows for examination of the individual chromosomes by microscopy. Prior to microscopy, the metaphase chromosomes are fixated on a glass slide and stained by chromatin staining to visualize each chromosome's characteristic banding pattern. Using this method, chromosomal abnormalities can be detected at a resolution of approximately 5-10

Mb. Karyotyping has been the gold standard in prenatal diagnosis of chromosomal abnormalities for many years, and has been performed on the majority of fetuses included in **Paper I-IV.** In all cases, at least 11 metaphase nuclei stained with quinacrine (Q) or Giemsa (G) banding have been analyzed. In the context of stillbirth, *i.e.* the area of focus in **Paper I and II**, karyotyping is prone to failure as the fetal tissue has in many cases started to macerate, resulting in unsuccessful cell culturing.

Quantitative fluorescence-polymerase chain reaction (QF-PCR) is a swift and easy way to detect aneuploidies of a limited number of chromosomes, usually chromosome 13, 18, 21, X and Y. The method is based on analysis of short tandem repeats (STRs), *i.e.* short fragments of DNA that are highly variable in size between individuals. By analyzing STRs located on the selected chromosomes, the number of chromosomes can be established. Using this method, only numerical aberrations involving the selected chromosomes can be identified, and no structural variants can be detected. However, it is a good complement to conventional karyotyping as it is not dependent on culturing of cells. In **Paper I,** QF-PCR was performed in all cases where the karyotype analysis had failed, and in **Paper III** it was used to analyze samples from fetuses where no clinical chromosome analysis had been performed previously.

3.3 CHROMOSOMAL MICROARRAY (CMA)

During the last decade, methods have been developed to identify chromosomal abnormalities at a higher resolution than conventional karyotyping and QF-PCR. Among these methods are the chromosomal microarrays (CMAs), such as array-based comparative genomic hybridization (array-CGH). The high resolution of these methods enables detection of CNVs (deletions and duplications) even at the level of single exons of known disease genes in targeted array formats. Patient and reference DNA are labeled with different fluorophores (commonly the cyanine dyes Cy3 and Cy5), and thereafter simultaneously hybridized to oligonucleotide probes printed on a microarray glass slide. The probes are designed to hybridize to DNA fragments located in the genomic areas of interest, which can either be evenly distributed across the complete genome, or in specifically targeted areas. Following hybridization, the slide is washed and scanned, and the fluorescent signals from both dyes are measured. A global normalization of the total signal from each dye is performed, whereupon the signals from each probe is measured and a log2 ratio between the signals is calculated. The signal intensity will be proportional to the concentration of DNA, and therefore differences in copy number between patient and reference DNA can be detected (Figure 2). Hence, the method can only detect unbalanced rearrangements, and thus not *e.g.* inversions or balanced translocations. However, the majority of these events are unlikely to cause a clinical phenotype in the carrier.

The resolution of the analysis is directly corresponding to the number and distribution of probes. In **Paper I**, an 180K oligonucleotide array with evenly distributed whole-genome coverage (Oxford Gene Technology), with a practical resolution of 20-50 kilobases (kb, thousands of basepairs), was used in the analysis. The same platform was also used to

investigate a sample which gave an unexpected result in **Paper IV**. The criterion for considering an aberration for analysis was that at least three consecutive probes had log2 ratios outside the normal range, where the threshold was 0.35 for duplications and -0.65 for deletions. For the detection of mosaic aberrations, a threshold of +/-0.1 was used with a 5 Mb cutoff. Aberrations were detected using a circular binary segmentation (CBS) aberration detection algorithm and additional manual interpretation/curation.

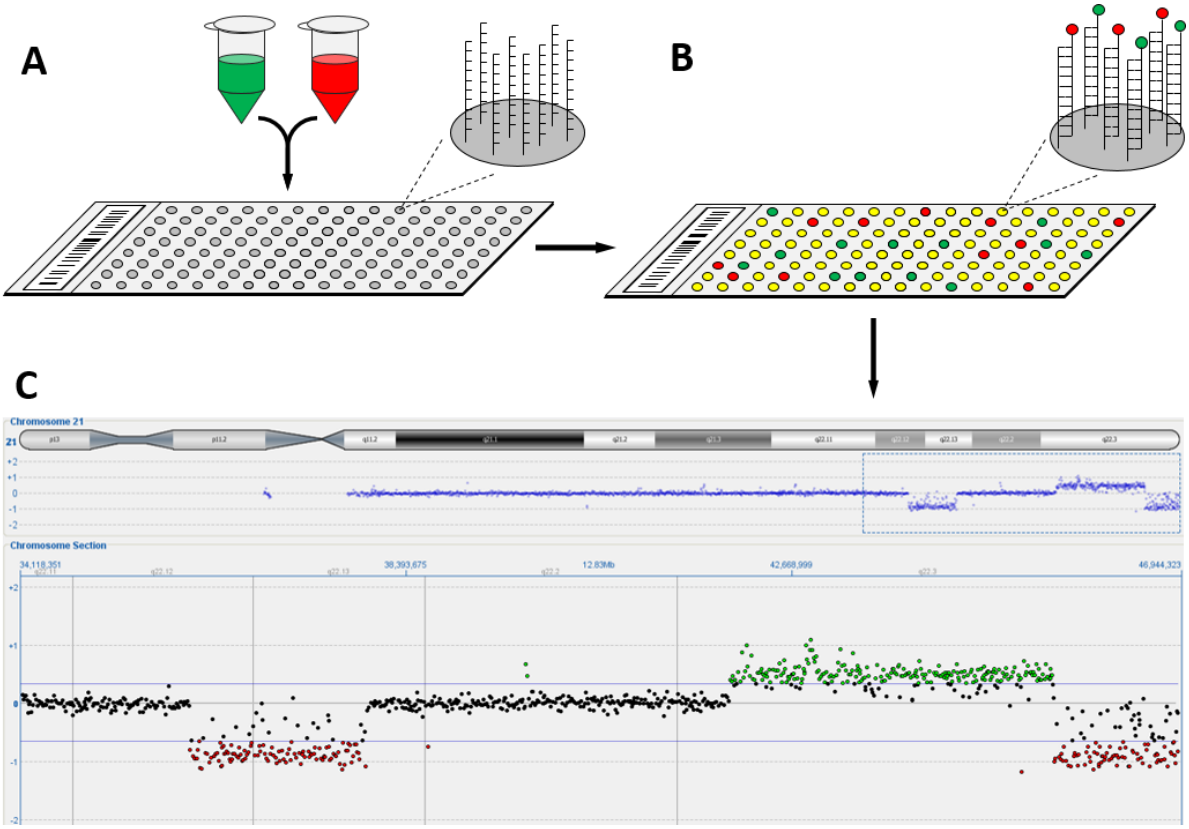


Figure 2. The principle of array-CGH. Patient and reference DNA are labeled with different fluorescent dyes and are thereafter allowed to simultaneously hybridize to oligonucleotide probes printed in spots on a microarray glass slide (A). Following hybridization, the color of each spot reflects the ratio between patient and reference DNA (B). The array slide is scanned, and the fluorescent signal for each dye is measured. The signal intensities are translated into a log2 ratio between patient and reference DNA, which is used in the analysis (C).

3.4 ANALYSIS OF CMA DATA

The interpretation of CNVs identified by CMA might be a challenging task, as their clinical relevance is often not obvious. A CNV can be considered pathogenic if it overlaps with a known syndrome region or genes known to cause disease. However, the challenge is that most CNVs are benign normal variants that are found in the general population. It has been estimated that approximately 12% of the human genome is covered by CNVs[76], but the proportion is likely higher as the definition was recently updated. The traditional definition of a CNV is that it should be at least 1 kb in size, but according to the new definition a CNV can be as small as 50 bp[77]. In cases where an identified CNV neither overlaps with a known

syndrome or disease region, nor a known normal variant, three major considerations are normally taken into account when trying to interpret its clinical relevance: inheritance, size and gene content. If a CNV is inherited from an unaffected parent, it is most likely benign. Studies suggest than more than 99% of benign CNVs are inherited, and that the vast majority of these are smaller than 500 kb. In contrast, most pathogenic CNVs are of *de novo* origin and larger than 1 Mb[78]. In general, CNVs affecting many genes are more likely to result in a clinical phenotype compared with those only affecting a few, as well as that loss of genes, *i.e.* a deletion, is generally more likely to be harmful than extra copies, *i.e.* a duplication, of them. However, for many of the recurrent microdeletion syndromes, a duplication of the same region also leads to a recognized syndrome. In **Paper I**, interpretation of the identified CNVs was complicated by the fact that no parental samples were available. Inheritance could therefore not be taken into consideration.

Classification of CNVs, from benign to pathogenic, was performed in accordance to previously described guidelines[79], with minor modifications. Briefly, identified CNVs were compared to published data sets, *e.g.* the CNV morbidity map described by Cooper *et al* in 2011[38], and databases such as the Database of Genomic Variants (DGV, http://projects.tcag.ca/variation), the International Standards for Cytogenomic Arrays Consortium (ISCA, http://dbsearch.clinicalgenome.org/), and the Database of Genomic Variation and Phenotype in Humans using Ensembl Resources (DECIPHER, https://decipher.sanger.ac.uk/). Also in-house data of postnatal CMA results from the Department of Clinical Genetics (including ~2,500 samples, mainly from patients with intellectual disability and/or congenital malformations) and data from 1,100 healthy blood donors were used for comparison. In addition, factors such as size, copy number status, and gene content were considered.

3.5 HALOPLEX LIBRARY PREPARATION

Recently, technologies have emerged that enable high throughput DNA sequencing. These techniques are collectively known as massive parallel sequencing (MPS) or next generation sequencing (NGS). Prior to the sequencing itself, a sequencing library that contains the DNA fragments of interest must be prepared. Different approaches can be used to fulfill this task. One option is to sequence the whole genome (whole genome sequencing, WGS) or all coding parts of a genome, *i.e.* the exome (whole exome sequencing, WES). However, in cases where a limited number of genes are of interest, library preparation of gene panels might be the preferable choice.

In **Paper II**, a gene panel containing 79 genes involved in heart disease and heart development was utilized, in order to study the potential role of heart disease in stillbirth. The genes were selected according to available literature, and were reported to be associated with channelopathies, cardiomyopathies or congenital heart defects (CHD). The Agilent HaloPlex target enrichment system was used to prepare libraries containing the exons and exon-intron boundaries of the selected genes. The outline of the method is displayed in Figure 3. Genomic

DNA is cleaved by using a cocktail of restriction enzymes to obtain short DNA fragments. Following restriction, a multitude of biotinylated probes are added to the DNA. The probes include DNA sequences that will specifically hybridize to the DNA of interest. By making use of the high affinity between biotin and streptavidin, the DNA-probe complexes are captured by using streptavidin-coated magnetic beads. The captured DNA fragments are amplified by polymerase chain reaction (PCR) to obtain multiple copies, which after purification is ready for sequencing.

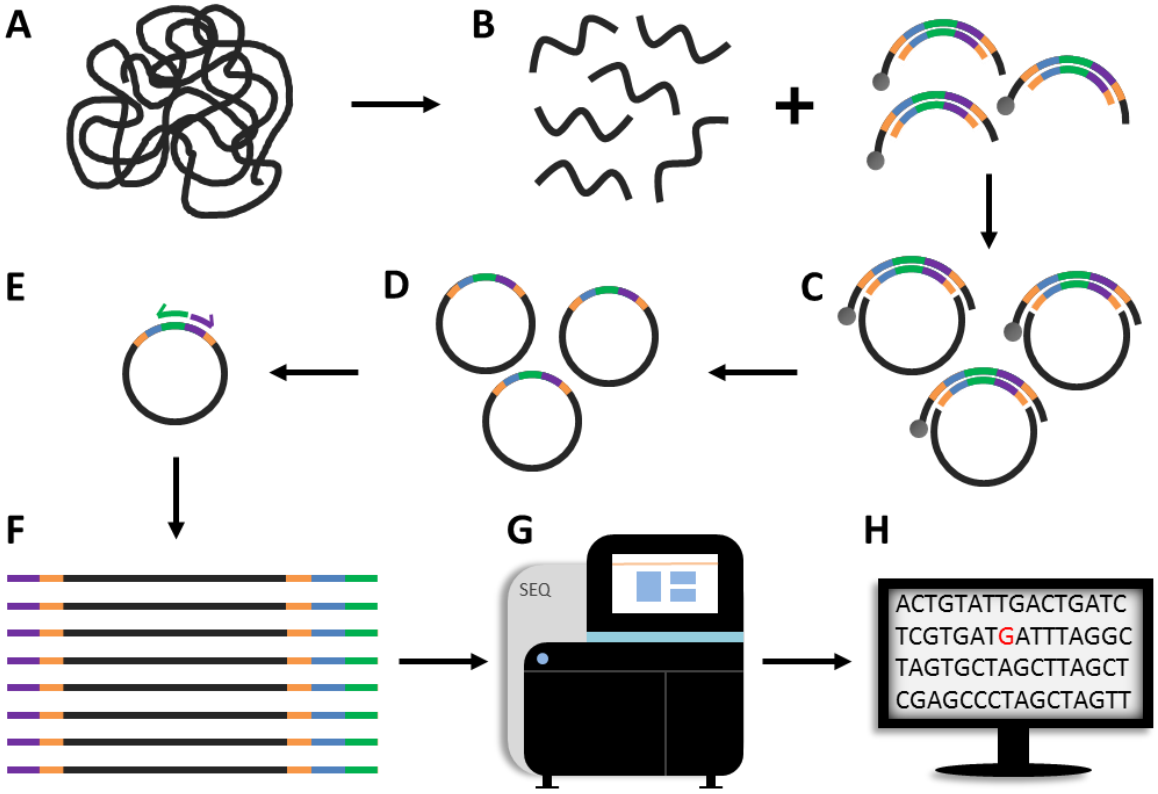


Figure 3. The principle of HaloPlex. Genomic DNA is fragmented using a cocktail of restriction enzymes (A). Biotinylated HaloPlex probes are added to the short DNA fragments (B). The probes contain complementary sequences to the DNA of interest (black), and will therefore hybridize to form circular DNA-probe complexes (C). The complexes are captured by using streptavidin-coated magnetic beads, whereupon the probes are ligated to the DNA (D). By using primers complementary to incorporated probe sequences (green and purple) (E), the DNA circles are amplified by PCR (F). The amplified library is sequenced (G), and analysis of identified nucleotide variants is performed (H).

3.6 AMPLIFICATION-FREE NIPT LIBRARY PREPARATION

Cell-free DNA (cfDNA) has some characteristic properties. It consists of short fragments, typically 160-200 bp, and is partially single-stranded. In that sense, it resembles ancient DNA. In **Paper IV**, a library preparation protocol originally created for ancient DNA[80,81] was modified to allow for amplification-free NIPT. Current other MPS-based NIPT methods include amplification of the DNA by PCR. A problem with PCR is that it inevitably leads to a bias due to some DNA fragments being amplified more efficiently than others. By

eliminating the PCR bias, the accuracy of NIPT could potentially increase. The amplification-free protocol was evaluated on 31 clinical samples, of which 15 had confirmed trisomies. The blood samples had been donated from women with high risk pregnancies according to FCT. The samples were centrifuged shortly after blood draw to collect the plasma, whereupon cfDNA was extracted.

As opposed to the HaloPlex library preparation, there is no need for restriction enzyme treatment in this protocol, as the cfDNA is already fragmented. Instead, the cfDNA is immediately denatured and dephosphorylated, and a biotinylated linker is ligated directly to each DNA fragment. The DNA-linker complexes are bound to streptavidin-coated magnetic beads, whereupon a single-stranded adaptor is added. The adaptor is complementary to the linker and will therefore hybridize to the DNA-linker complexes. The adaptor is extended by a polymerase to create a complementary strand to the cfDNA, which thereby becomes double-stranded. A second, double-stranded, adaptor is added and ligated to the DNA by blunt end ligation. The DNA is thereafter denatured, and the free DNA strand, *i.e.* the strand that is not bound to the magnetic bead, is collected for sequencing.

3.7 MASSIVE PARALLEL SEQUENCING (MPS)

The technology used to sequence the libraries yielded by both HaloPlex and the amplification-free NIPT protocol was Illumina's sequencing-by-synthesis method, utilizing reversible termination chemistry. The DNA library to be sequenced is denatured and added to a lane in a flowcell. The lane is covered by covalently bound adaptors that will hybridize to the library fragments. The adaptors also work as PCR primers, used to enable amplification of the hybridized library fragments to create dense clusters containing multiple identical copies. The cluster generation is required to increase signal intensity. Following cluster generation, the DNA fragments are denatured once more to enable the sequencing reaction. A universal sequencing primer is added, and extended by one nucleotide at a time. To ensure that only one nucleotide is incorporated in each cycle, they are modified with a terminator that blocks further elongation. Additionally, each nucleotide is labeled with a specific fluorophore. Following incorporation of a nucleotide, the fluorescent signal from each cluster is recorded, whereupon the terminator is cleaved off to allow for a new cycle of nucleotide incorporation[82].

3.8 ANALYSIS OF MPS DATA

Both the HaloPlex and the amplification-free NIPT libraries were made for the purpose of MPS. However, the desired data was completely different. In the case of HaloPlex, the sequence itself is of interest, and a deep coverage of the selected genes was required to identify sequence variants with high accuracy. Conversely, NIPT is a counting assay, which means that as long as the reads can be mapped to the reference genome, the sequence itself is not important, but rather the number of sequenced DNA fragments.

3.8.1 HaloPlex data analysis

A custom script pipeline originally created for analysis of HaloPlex sequencing data generated from the Illumina MiSeq instrument, was utilized to identify sequence variants in the 79 genes associated with heart disease and development included in the gene panel. Previously published data have shown that the custom script yields results of higher quality than other pipelines used as common practice for analysis of MPS data[83]. The custom script was originally created in order to achieve a faster analysis procedure, as well as to remove reads of low quality.

The basis of the original custom script is that MiSeq HaloPlex sequence reads are generally of very high quality. The first step is to sort the sequence reads, and map them against each other. Reads of poor quality are expected to vary between themselves in a random fashion, due to sequencing errors. By only taking identical reads recurring ≥3 times in the data into account, reads of poor quality are sorted out. For each group of identical reads, one representative is mapped against the human genome (version GRCh37/hg19). By aligning only one representative for each read instead of thousands of identical copies, a vast amount of computer times is saved, resulting in a total run time of only a few minutes per sample, including computation of coverage and variant calling. However, in **Paper II,** the NextSeq instrument was used, requiring modifications of the protocol, due to that reads generated from NextSeq have a higher frequency of erroneous base calls. Instead of removing reads with low quality bases, the low quality base calls were N-substituted and paired-end analysis was performed without pooling of identical read pairs. Variants were called when the ratio of reads including the variant divided by the total number of reads at the specific position was ≥0.25 for SNVs and ≥0.12 for insertions and deletions.

The identified variants were filtered to exclude variants occurring at a frequency of >1% in population databases, as were synonymous variants, and intronic variants that were not predicted to affect splicing. The remaining variants were individually evaluated based on available literature, type of variant (missense, loss-of-function variant), entries in the Human Gene Mutation Database (www.hgmd.org), ClinVar (www.ncbi.nlm.nih.gov/clinvar/) and dbSNP (www.ncbi.nlm.nih.gov/SNP/), and evolutionary conservation. Additionally, the Combined Annotation Dependent Depletion (CADD) score was used to assess the deleteriousness of the variants[84]. Compared with other tools used to predict functional effects of genetic variants, CADD has the advantage that it combines several different annotations to create a single score. The scaled CADD score, which was used in this study, relates the variant of interest to all possible theoretical variants in the genome, whereupon a logarithmic score is returned. A score of 20 indicates that the variant is among the 1% most deleterious variants in the genome, 30 indicates that it is among the 0.1% most deleterious variants, and so on.

A variant was classified as pathogenic if it was either a loss-of-function (LoF) variant, or a missense variant where previously published data suggested an association between the

variant and the disease of interest. Conversely, missense variants for which no data supporting pathogenicity had been published, and which additionally were located at poorly conserved positions and had a low CADD score, were considered likely benign and excluded from further evaluation. Missense variants which did not fall into either of these categories were considered to be of unknown clinical significance.

3.8.2 Amplification-free NIPT data processing

The amplification-free NIPT protocol was used on 31 samples, of which 15 had confirmed trisomies. Four female samples were used for normalization. One of the normalization samples was used to create windows containing 10,000 amplification-free reads (AFRs) in each. The same windows were applied on the remaining normalization samples, and the mean number of AFRs in each window was calculated and used for normalization of the rest of the samples. The number of reads per window was centered around one, based on the average number of reads per window on all chromosomes, with the exception of sex chromosomes, potential trisomy chromosomes 13, 18 and 21, as well as chromosome 16 and 19 that sometimes showed an irregular number of reads. Loess local regression was performed to correct for residual GC bias, and the 10% windows with lowest and highest normalized read counts (NRCs) were excluded to get rid of outliers.

3.9 QUESTIONNAIRE

NIPT was introduced at the Department of Clinical Genetics, Karolinska University Hospital, in June 2015. Thereby it became the first NIPT service available via national health care in the Nordic countries. When introducing a new method, it is of value to study the interest, potential uptake, and how the method may be accepted by the prospective users. In **Paper V**, we developed a questionnaire to be answered by pregnant women, covering awareness, attitudes, preferences about risk information and decision-making concerning prenatal examinations, with emphasis on NIPT. Additionally, attitudes towards having a child with a chromosomal abnormality were studied, as well as the women's self-perceived likelihood for that event to occur. The questionnaire contained background questions as well as multiple-choice questions and Likert scales covering the different topics. Women were recruited to fill in the questionnaire at nine maternity clinics located in different areas of Stockholm, selected to represent a broad variety in socioeconomic status of the participants.

3.10 STATISTICAL ANALYSES

Various statistical analyses have been used in the studies included in this thesis, of which the majority has been performed in order to identify significant differences in proportions of various test statistics between different cohorts or subgroups. In **Paper I, II and V,** Fisher's exact test has been performed in cases where there are two different outcomes, whereas the chi^2 test has been used in cases of multiple outcomes. Because of the study design,

McNemar's test for matched case-control studies was performed to identify differences between subgroups in **Paper III**.

In **Paper II,** the frequency of SNVs identified in the 290 investigated stillbirth cases was compared with variant frequency data reported by the Exome Aggregation Consortium (ExAC, www.exac.broadinstitute.org), which includes exome sequencing data from more than 60,000 individuals. The proportion of pathogenic SNVs was compared between the stillbirth cohort and ExAC by using Fisher's exact test as described above. However, that calculation was based on the SNVs that actually had been identified in the study cohort, and in order to get an approximation of the proportion of pathogenic SNVs in relation to the total number of SNVs in the different cohorts, a Monte Carlo permutation test was performed. The test included 10,000 iterations, in which groups of 50 variants from each cohort were randomly drawn. The number of pathogenic SNVs in each group was counted, whereupon the mean proportion of pathogenic SNVs across the 10,000 iterations was calculated. The difference between means of proportion of pathogenic SNVs for the stillbirth cohort and ExAC was thereafter calculated, and a 95% confidence interval (CI) based on the binomial distribution was established in order to investigate the null hypothesis that there was no difference in means. The obtained proportions of pathogenic SNVs in both the study cohort and ExAC was used to calculate the expected proportion of pathogenic SNVs if another cohort of cases would have been drawn from the same binomial distributions.

Results from the different analyses were considered statistically significant when $p<0.05$.

4 RESULTS AND DISCUSSION

4.1 CHROMOSOMAL ABNORMALITIES IN STILLBIRTH (PAPER I)

Using traditional karyotyping or QF-PCR, chromosomal abnormalities were identified in 7.5% (n=36) of the 481 stillbirth cases included in **Paper I**. This is consistent with what has been published previously[6]. The distribution of chromosomal abnormalities is displayed in Figure 4.

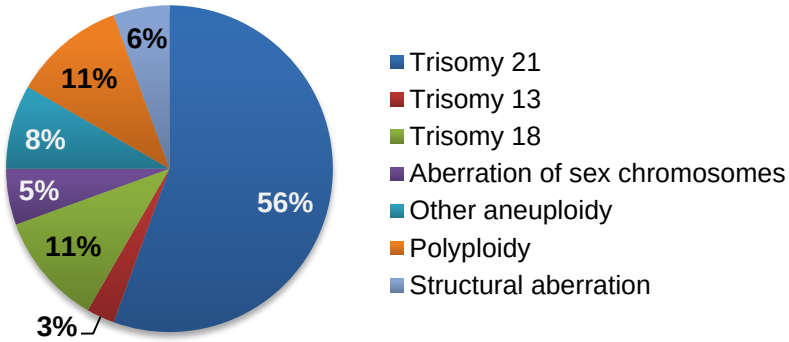


Figure 4. Chromosomal abnormalities in stillbirth. Distribution of aberrations identified in 36 out of 481 stillbirth cases (7.5%) by using either karyotyping or QF-PCR.

Analysis with CMA of 90 cases additionally identified two known microdeletion/-duplication syndromes, one aberration disrupting a known disease-gene associated with the observed phenotype in the particular stillbirth case, and 29 variants of unknown significance (VOUS), of which none could have been detected using conventional karyotype analysis. One of the known syndrome associated aberrations was a 3.7 Mb deletion located on chromosome 17, associated with Smith-Magenis syndrome (Figure 5). Features of Smith-Magenis syndrome include characteristic facial features, intellectual disability, behavioral problems and sleep disturbances, as well as feeding difficulties and failure to thrive[85]. This aberration may have contributed to the fetal demise, as it affects several neurological and developmental processes. The second syndrome associated aberration was a 523 kb duplication located on chromosome 16, associated with the 16p11.2 duplication syndrome (Figure 5). Aberrations in this region are identified in up to 1% of patients with autism spectrum disorders[86,87], but also other neuropsychiatric disorders, such as schizophrenia[88] and epilepsy[89]. The aberration is unlikely to have caused the fetal demise, however detecting aberrations of this kind may be of value for the extended family as they are often inherited by an unaffected or only slightly affected parent, and may cause symptoms in a future child[86]. The aberration affecting a known disease-gene was a small duplication, 23 kb in size, located on chromosome 14 (Figure 5). The duplication likely disrupts the genes *MYH6* and *MYH7*. Studies have shown that defects in *MYH6* can cause atrial septal defect[90,91] – a congenital heart defect (CHD) that was identified in the stillborn fetus.

In addition to the known syndrome- or disease associated CNVs, 29 VOUS were identified. Nine of the VOUS, in eight stillbirth cases, were 500 kb or larger. As mentioned previously, reports have suggested that more than 99% of benign variants are inherited, and that the majority of them are smaller than 500 kb[78]. Based on this, the VOUS larger than 500 kb identified in our cohort are the most likely to be pathogenic. Unfortunately, the inheritance pattern could not be determined as parental samples were not available. However, one of the smallest aberrations that were detected was most probably pathogenic, *i.e.* the duplication disrupting *MYH6.* This illustrates the importance of evaluating all aberrations, regardless of size.

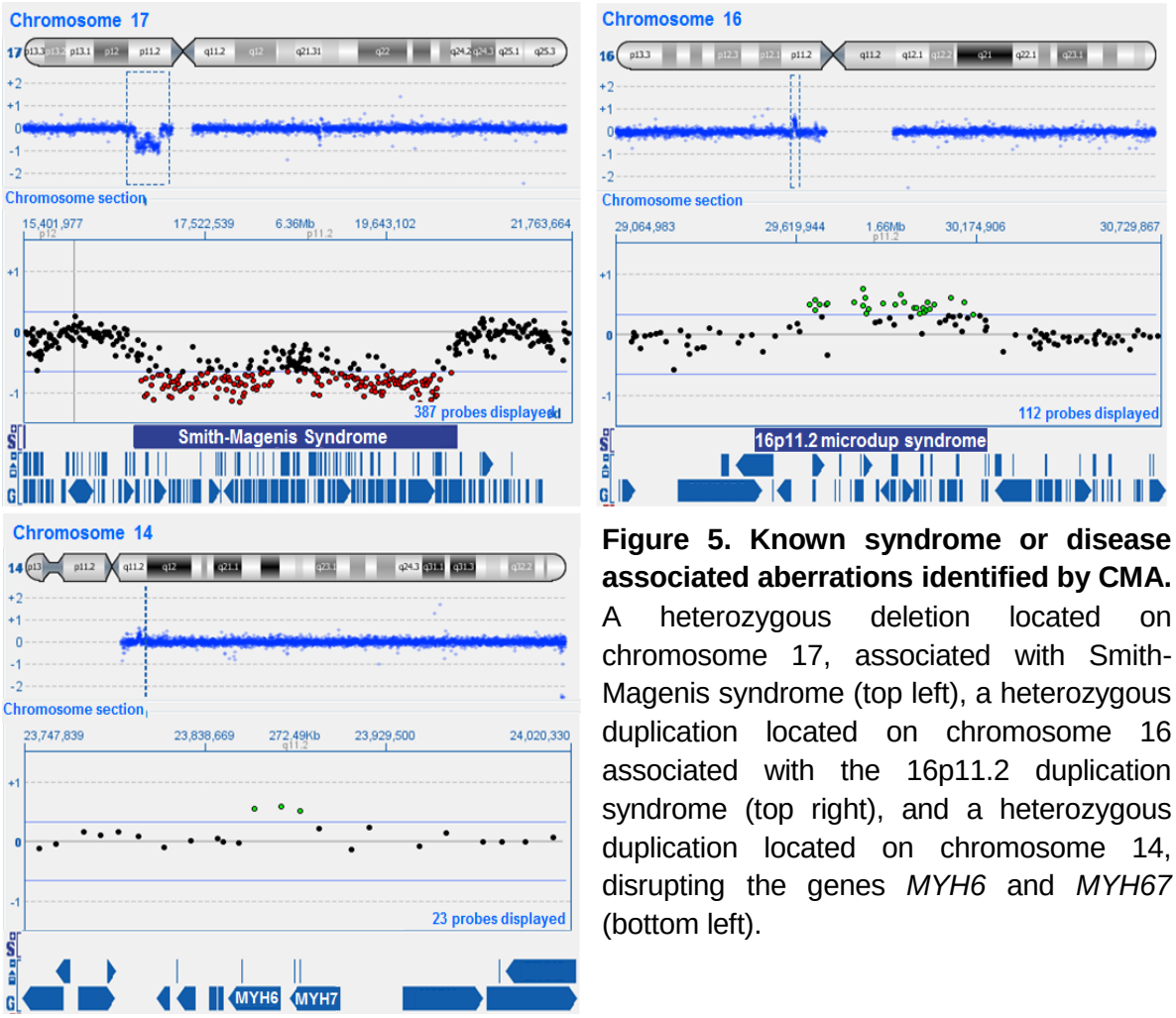


Figure 5. Known syndrome or disease associated aberrations identified by CMA. A heterozygous deletion located on chromosome 17, associated with Smith-Magenis syndrome (top left), a heterozygous duplication located on chromosome 16 associated with the 16p11.2 duplication syndrome (top right), and a heterozygous duplication located on chromosome 14, disrupting the genes *MYH6* and *MYH67* (bottom left).

4.2 PERFORMANCE BY DIFFERENT CHROMOSOME ANALYSIS METHODS IN STILLBIRTH INVESTIGATION (PAPER I)

Using conventional karyotyping, results were obtained in 391 out of the 481 of the stillbirth cases included in the study (81.3%). QF-PCR yielded a result in 86 of the remaining cases. In four cases, both karyotyping and QF-PCR failed. Analysis with CMA on 90 samples yielded results of excellent quality in 100% of the cases, *i.e.* a significantly higher success rate than karyotyping ($p<0.001$). Excellent was defined as a derivative log ratio (DLR)

spread below 0.15, according to recommendations from the manufacturer. Interestingly, the excellent results were true also for DNA samples which were highly degraded according to results from gel electrophoresis.

Chromosomal aberrations were identified in 7.5% of the stillbirth cases by using karyotype analysis. Based on the aberrations identified in this cohort, QF-PCR would have yielded a chromosomal aberration detection frequency of 5.8% if it had been used on all samples. Our results suggest that comparative genomic hybridization based CMA could potentially increase the detection frequency in stillbirth from 7.5% to 10-20%, based on an additional detection of 3% known syndromes/monogenic disorders (lower span), and the detection of VOUS ≥500 kb (higher span). However, array-CGH would fail to detect the polyploidies as the increase in chromosome material is even across the whole genome. Using a standard reference sample, this type of array will only indicate the presence of potential polyploidy if there is a deviating ratio between the sex chromosomes, *e.g.* 69,XXY, which was not the case for any of the polyploidies identified in this cohort.

4.3 ASSOCIATION BETWEEN MATERNAL AGE AND STILLBIRTH (PAPER I)

An association between advanced maternal age and stillbirth has been shown in several previous studies[92-95], and the same pattern was true also in our material. The proportion of stillbirths in different maternal age groups corresponded well to the proportion of child births by the same age groups in Stockholm, except for women ≥40 years of age, in which stillbirth was significantly overrepresented ($p<0.001$). Additionally, the proportion of stillbirth cases with chromosomal abnormalities was significantly higher in this age group (19.5%, $p=0.006$). This is not surprising as it is well known that the risk of chromosomal abnormalities increases with maternal age. Interestingly, the lowest proportion of aberrations, 2.9%, was identified in women of 35-39 years of age.

4.4 PATHOGENIC VARIANTS ASSOCIATED WITH HEART DISEASE IDENTIFIED IN STILLBIRTH (PAPER II)

We analyzed 290 stillbirth cases without known chromosomal abnormalities with a customized HaloPlex gene panel, including 79 genes involved in heart disease and heart development. Of the investigated cases, 62 (21.4%) harbored one (n=58) or two (n=4) variants with evidence supporting pathogenicity, *i.e.* LoF variants (nonsense, frameshift or splice site substitutions), evidence from functional studies, or previous identification of the variants in affected individuals. The proportion of individuals harboring the same variants reported in ExAC was significantly lower, 7.94% ($p<0.001$, Figure 6). Fifty-three percent of the identified pathogenic variants were located in channelopathy genes, 24% in cardiomyopathy genes, and 23% in genes mainly associated with congenital heart defects (CHD).

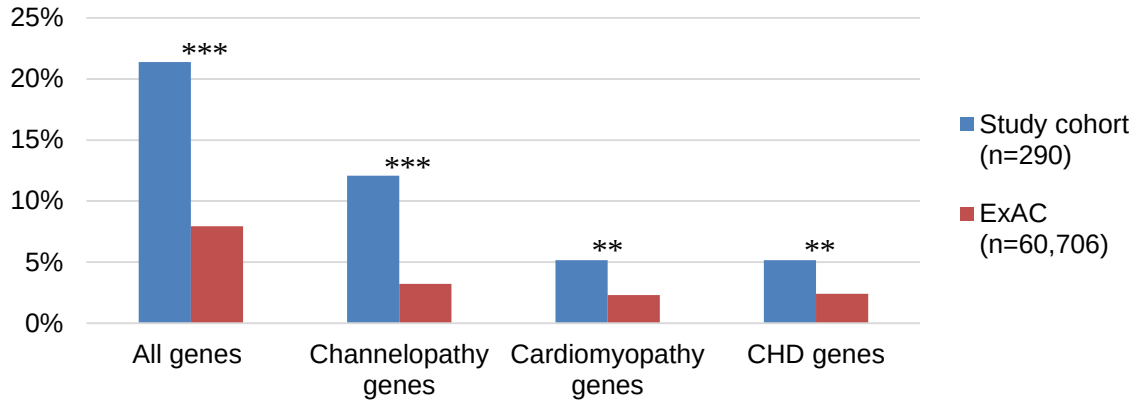


Figure 6. Proportions of individuals with pathogenic SNVs in the study cohort, compared with the corresponding proportions in ExAC for the same variants. ***=p<0.001, **=p<0.01

The genes included in the panel, as well as the specific genes in which pathogenic SNVs were identified, are displayed in Table 1. In addition to the variants with evidence supporting pathogenicity, 174 missense variants of unknown significance were identified in 125 stillbirth cases.

Table 1. Genes included in the customized HaloPlex gene panel, their associated diseases, and identified pathogenic SNVs.

Category	Disease	Genes with identified pathogenic SNVs (n=number of variants)	Genes with no identified pathogenic SNVs
Cardiomyopathies	ARVC	*DSG2* (n=2), *DSP* (n=2), *PKP2* (n=1)	*DSC2, JUP, TGFB3, TMEM43*
	DCM	*ABCC9* (n=1), *BAG3* (n=1), *DES* (n=1), *NEBL* (n=1), *NEXN* (n=1), *TNNI3* (n=1)	*ACTN2, ANKRD1, DMD, DNAJC19, FHL2, LDB3, LMNA, PLN, RBM20, TAZ, TCAP, TMPO, TNNC1*
	HCM	*CSRP3* (n=1), *MYBPC3* (n=2), *MYH7* (n=1), *TTN* (n=1)	*ACTC1, BRAF, CALR3, CAV3, GLA, JPH2, LAMP2, MYL2, MYL3, MYLK2, MYOZ2, PRKAG2, TNNT2, TPM1, VCL*
Channelopathies	LVNC		*DTNA, MIB1*
	BrS	*CACNB2* (n=1), *GPD1L* (n=4), *HCN4* (n=5), *KCNJ8* (n=1), *TRPM4* (n=4)	*CACNA1C, CACNA2D1, KCNE3, SCN1B, SCN3B, SCN4B*
	CPVT	*CASQ2* (n=1), *RYR2* (n=1)	
	LQTS	*ANK2* (n=5), *KCNH2* (n=3), *KCNQ1* (n=5), *SCN5A* (n=3)	*AKAP9, KCNE1, KCNE2, KCNJ2, KCNJ5, SNTA1*
CHD		*GATA4* (n=9), *GATA5* (n=5), *NOTCH1* (n=1)	*FBN2, FGF16, HRAS, MAP2K2, MYH6, TBX5*

ARVC: arrhythmogenic right ventricular cardiomyopathy, BrS: Brugada syndrome, CHD: congenital heart defects, CPVT: catecholaminergic polymorphic ventricular tachycardia, DCM: dilated cardiomyopathy, HCM: hypertrophic cardiomyopathy, LQTS: long QT syndrome, LVNC: left ventricular non-compaction cardiomyopathy

Genes in which at least five pathogenic SNVs were identified were searched in ExAC, and all reported missense and LoF variants were systematically searched for in the ClinVar database, in order to get a crude approximation of how common pathogenic SNVs are in the general population for these genes. ClinVar is a public archive containing reports of the relationships between human variations and phenotypes, together with supporting evidence. The results showed a significantly higher total number of observations of pathogenic alleles in *KCNQ1* in relation to wild type alleles in the study cohort compared with ExAC (0.86% vs. 0.29%, p=0.0289), whereas no significant difference was seen for the other genes analyzed (0.86% vs. 1.25%, p=0.5709 for *ANK2*, 1.55% vs. 0.81%, p=0.5932 for *GATA4*, and 0.86% vs. 0.41%, p=0.0908 for *GATA5*, respectively).

Previous studies have mainly focused on stillbirth in association with channelopathies, and especially long QT syndrome (LQTS). LQTS affects cardiac ion channels, and is characterized by a prolonged Q-T interval on echocardiogram. The condition is a common cause of sudden death postnatally[96], and is diagnosed in up to 9.5% of infant death syndrome cases[97]. Crotti *et al* studied 91 stillbirth cases for SNVs in the most common LQTS susceptibility genes, *i.e. KCNQ1, KCNH2* and *SCN5A.* They identified putative pathogenic variants in 3.3% of their cases[98], and the proportion of putative pathogenic SNVs for the same genes in our study was very similar; 3.7% (n=11). One variant, *KCNQ1*, p.Arg397Trp, was identified in both studies, and it has been shown to cause a significant reduction in current densities across the potassium channel encoded by *KCNQ1*, compared with the wild type channel[98]. Furthermore, the total number of pathogenic alleles in *KCNQ1* observed in our cohort was significantly higher than in ExAC (p=0.0289), which supports that SNVs in this gene might play a role in stillbirth. In addition to the most common LQTS genes, five cases in our cohort harbored SNVs in *ANK2*. However, the frequency of pathogenic SNVs in *ANK2* identified in our cohort did not differ significantly to the frequency in ExAC. This might reflect that *ANK2* is mainly associated with LQTS type 4, which phenotypically is highly variable and may go unnoticed throughout life[99].

Pathogenic SNVs in genes associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) were identified in four cases (1.4%), of which three were located in *RYR2* and one in *CASQ2*. CPVT is one of the most severe cardiac channelopathies, and is characterized by ventricular arrhythmias causing syncope, cardiac arrest and sudden cardiac death, predominantly in young patients including infants[100,101]. Therefore, these SNVs are good candidates for being associated with stillbirth. Additionally, SNVs associated with Brugada syndrome (BrS) were identified in 15 cases (5.2%). BrS has mainly been described in association with sudden death in adults previously, and therefore its role in stillbirth is difficult to interpret. However, two SNVs, located in *GPD1L* and *HCN4*, are worth highlighting. The *GPD1L* variant p.Ile124Val was found in four cases in our cohort, resulting in a MAF of 0.69%, which is more than 3 times higher than the MAF of 0.21% reported in ExAC (p=0.0333). The *HCN4* variant p.Val759Ile was identified in five cases, resulting in a MAF of 0.86%, *i.e.* a 2.5 fold increase compared with the corresponding number, 0.35%, in ExAC (p=0.0570). Both these variants have been associated with sudden infant death

syndrome (SIDS) in previous studies[102,103]. Although the latter of the variants did not quite reach statistical significance, the increased frequency of these SNVs in our cohort might indicate that they are risk factors for stillbirth as well as SIDS.

SNVs with evidence supporting pathogenicity in genes associated with cardiomyopathies were identified in 15 cases (5.2%). Cardiomyopathies, *i.e.* disorders of the heart muscle, are impairments of the ability of the myocardium to contract, which can result in heart failure[104]. In general, cardiomyopathies are progressive disorders which are not detected during the early years of life, and have therefore not been studied in association with stillbirth previously. However, studies suggest that they might play a role in SIDS. Brion *et al* studied 286 SIDS cases for variants in genes associated with hypertrophic cardiomyopathy (HCM) and found variants with possibly damaging effects in 4% of the cases[105]. One of their identified SNVs, *MYBPC3*, p.Ala833Thr, was detected in two of our cases. Brion *et al* hypothesized that their identified variants might cause sudden cardiac death even in the absence of a cardiac phenotype, but they did also emphasize the possibility that the variants could be non-disease causing rare variants[105].

Congenital heart defects (CHD) is the group of malformations that is most common in live born infants[106,107], and is additionally the most frequent cause of infant death resulting from birth defects[108]. Fifteen cases in our cohort (5.2%) had variants in genes mainly associated with CHD *(i.e. GATA4, GATA5* and *NOTCH1).* Autopsy had been performed on 13 of these 15 cases, and the only heart malformation detected was an atrial septal defect in the fetus with the variant in *NOTCH1*; a gene that has mainly been associated with aortic valve anomalies[109]. Hence, the clinical significance of these SNVs is difficult to interpret. Additionally, the frequency of pathogenic SNVs identified in *GATA4* and *GATA5* in our cohort did not differ significantly from the frequencies reported by ExAC. However, variants in *GATA4* have also been associated with atrial fibrillation in addition to structural heart malformations[110].

In order to compare the proportion of pathogenic SNVs in the stillbirth cohort to ExAC across all 79 genes included in the gene panel, a Monte Carlo permutation test with 10,000 iterations was performed. In each iteration, 50 variants were randomly drawn from the stillbirth cohort and ExAC, and the average proportions of pathogenic SNVs were calculated. In the stillbirth cohort, the average proportion was 0.0351, whereas the corresponding proportion in ExAC was 0.0235. The difference in average proportions is hence 0.0117, with a 95% CI of [0.01, 0.0135]. As the confidence interval does not include 0, the null hypothesis, *i.e.* that the proportion does not differ between the stillbirth cohort and ExAC, can be rejected. The obtained proportions were used to calculate the expected proportion of pathogenic SNVs if new cohorts would be drawn from the same binomial distributions. If drawn from a population with similar characteristics as ExAC, the expected proportion would be 13.2%, with a 95% CI of [9.4%, 17.2%]. The proportion of pathogenic SNVs identified in our study, 21.4%, thus lies outside the expected range for a cohort similar to ExAC. If instead

a cohort would be drawn from a population similar to the stillbirth cohort included in the study, the expected proportion would be 19.3%, with a 95% CI of [14.8%, 23.8%].

Taken together, our findings indicate a modest but significantly increased frequency of pathogenic SNVs in stillbirth compared to what is reported by ExAC, and gives further support to the hypothesis that channelopathies, and potentially also cardiomyopathies and CHD, might be associated with stillbirth. Additional studies, where parental samples, comparable control groups, and functional analyses are included, are required to further establish an association between stillbirth and heart disease. Furthermore, because of the high frequency of variants of unknown significance, clear guidelines regarding how to handle these findings are necessary before the method is suitable for implementation in clinical practice.

4.5 CHROMOSOMAL ABNORMALITIES IN FETUSES WITH TISSUE CALCIFICATIONS (PAPER III)

The proportion of fetuses with tissue calcifications in the archives of the Department of Pathology was 5.3%, and the calcifications were mainly located in the liver (57%), but also in heart (13%), bowel (6%), or even multiple tissues (22%). Fetuses with calcifications showed a significantly higher frequency of chromosomal abnormalities compared with controls; 50% vs. 20% ($p<0.001$). The most frequent aberrations included trisomy 21 (33%), trisomy 18 (22%), and monosomy X (18%). The distribution of chromosomal abnormalities was similar in controls with aberrations.

The fetuses were divided into different subgroups, based on gestational age, type of death, and tissue location of calcifications. Regarding gestational age, the highest proportion was seen in fetuses <14 gestational weeks (71%) and lowest in week ≥29 (17%). Regarding type of death, the highest proportion was seen among cases after induced termination (63%). This was expected because a common reason for pregnancy termination followed by autopsy is chromosomal abnormalities. The lowest frequency was observed in the stillbirth group (31%). However, note that this “low” proportion is more than three times higher than the overall proportion of chromosomal abnormalities in stillbirth, according to results in **Paper I.** No difference in proportions was observed in subgroups based on tissue location of calcifications.

The prevalence of fetal malformations was significantly higher in cases than in controls (72% vs. 50%, $p<0.001$). When divided into groups based on the presence of chromosomal abnormalities or not, the difference in malformation prevalence was only seen between cases and controls with chromosomal abnormalities (95% vs. 77%, $p=0.004$). The corresponding numbers in cases and controls without chromosomal abnormalities were 49% vs. 43% ($p=0.446$). It was expected that fetuses with chromosomal abnormalities would have a higher prevalence of malformations than those without, but the discrepancy in prevalence between cases and controls is notable. These results could indicate that calcification is a part of the

phenotypic spectrum caused by various chromosomal abnormalities, and is more likely to occur in fetuses with a more severe phenotype. When analyzed from the opposite direction, *i.e.* the proportion of chromosomal abnormalities in cases and controls with or without malformations, cases with malformations had a significantly higher proportion of chromosomal abnormalities compared with controls, (66% vs. 31%, $p<0.001$). Cases and controls without malformations showed equal proportions of chromosomal abnormalities (10% vs. 9%). In practice, our results suggest that if a fetus has a calcification and a malformation, the probability of a chromosomal abnormality is increased by more than double, compared with if the fetus has a malformation only.

4.6 PERFORMANCE OF AMPLIFICATION-FREE NIPT (PAPER IV)

The amplification-free NIPT method correctly identified all autosomal trisomies, which included one case of trisomy 13, four cases of trisomy 18, and eight cases of trisomy 21. Additionally, it could correctly determine the number of X chromosomes in both normal XX and XY samples, as well as in one case of X0 (Turner syndrome) and one case of XXY (Klinefelter syndrome). However, in one case for which standard cytogenetic testing had confirmed the presence of both trisomy 18 and XXY, NIPT failed to identify the additional X chromosome (Figure 7).

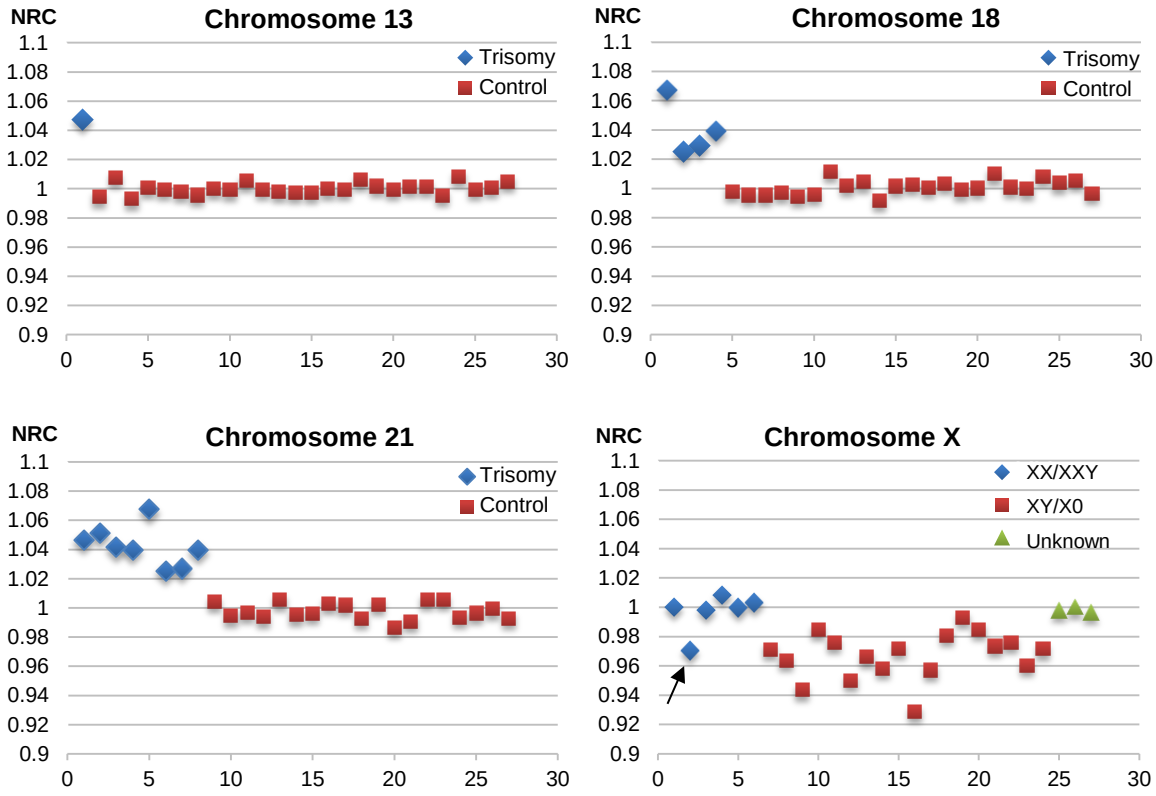


Figure 7. Results from amplification-free NIPT. The method could correctly identify all chromosomal abnormalities previously identified by karyotyping or QF-PCR, except for one case of XXY where NIPT failed to detect the extra X chromosome (arrow). NRC = normalized read count.

To understand why the additional X chromosome was not detected, lung tissue from the fetus was obtained from the Department of Pathology, Karolinska University Hospital. DNA was extracted followed by analyses by array-CGH and amplification-free sequencing. The additional analyses showed that the fetus actually was mosaic; 70% of the cells carried the extra X chromosome, while 30% carried normal male sex chromosomes (XY). The presence of trisomy 18 was confirmed in 100% of the cells by the additional analyses. The mosaicism probably explains why the additional X chromosome was not identified using the amplification-free protocol. As cffDNA mainly is derived from the placenta, it would have been optimal if placental DNA could have been analyzed, but unfortunately no such tissue was available. However, considering the mosaic status of the fetus, it is possible that the XY profile identified by NIPT reflected the placental status, rather than a technical error.

4.7 PREGNANT WOMEN'S ATTITUDES TOWARDS PRENATAL EXAMINATIONS WITH EMPHASIS ON NIPT (PAPER V)

4.7.1 Awareness, interest and information preferences regarding NIPT

Attitudes towards NIPT were studied among 1,003 women prior to the introduction of the method into Swedish healthcare. Although 60% of the women stated that they had heard about the method previously, 74% stated that they would like to use the method if available. This implies that a test with the characteristics of NIPT; *i.e.* both highly accurate and safe for the fetus, is very desirable by the pregnant population. Among the remaining women, 12% stated that they would not like to use the method, whereas 14% were not sure. Internationally, the interest in using NIPT has been shown to vary greatly between different countries, ranging from 51% in the Netherlands to 88% in the UK[111-113]. Based on that 64% of the women in Stockholm took FCT in 2014, the proportion of women accepting prenatal screening would potentially increase by at least 10% if FCT was substituted by NIPT. As of today, NIPT is only offered women classified as high risk by FCT, *i.e.* a risk exceeding 1:200.

Although most of the women seemed to have decided whether they wanted to use NIPT or not, a large majority stated that they would need information to facilitate their decision if to undergo the test. However, the proportion differed significantly between women who had stated that they wanted to use the test and the women who would not use it; 84% vs. 64%, respectively ($p<0.001$). This might indicate that women who are against chromosomal testing are more convinced about their choice. The most requested way to receive information was orally by the midwife (80%), followed by written information (49%), however often in combination.

Regarding which information the women would like to get if they indeed would use the test, yet again the answers differed significantly between women potentially willing to use NIPT and women who would not use the test. Out of the women potentially willing to use NIPT, 96% would like to know if the fetus had Down syndrome, whereas the corresponding

proportion among women not willing to use the test was only 48% (p<0.001). However, considerably more (69%) would like to know if the fetus had another, more severe chromosomal abnormality (Figure 8). Out of the women who would potentially use NIPT, 78% stated that they would be willing to pay by themselves if the cost would not be covered by the national health insurance. Most of them (74%) would pay approximately €50 to €100, whereas approximately 10% stated that they would pay €500 or more.

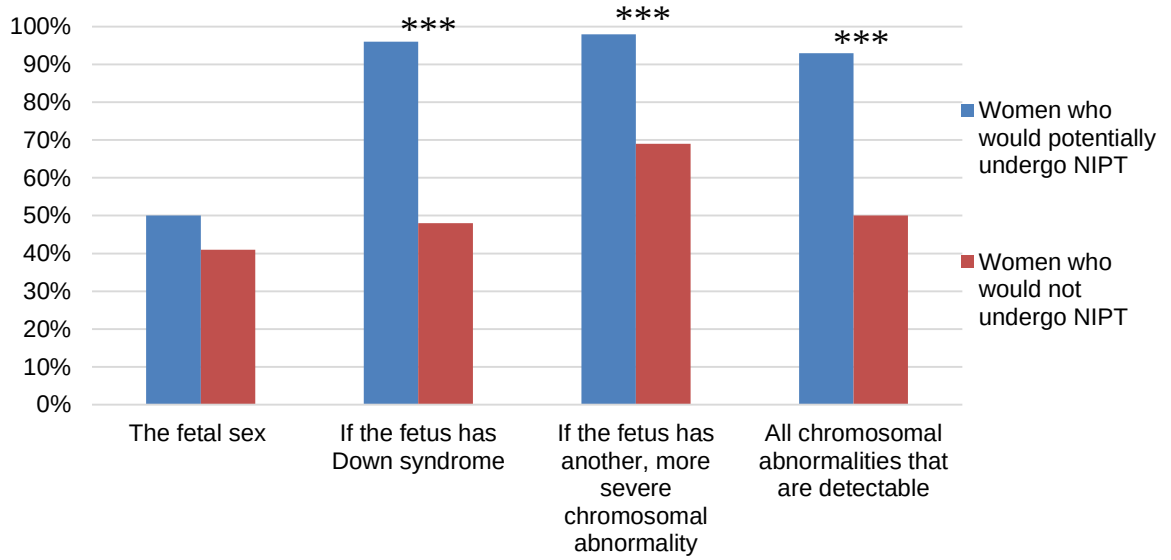


Figure 8. Desired information from a NIPT analysis. *** = p<0.001

4.7.2 Attitudes towards prenatal examinations in general

The overwhelming majority, 91%, considered examinations aiming to detect fetal abnormalities to be good. However, 27% perceived such examinations as frightening. Yet, the majority thought that the examinations are calming (69%), and that it is self-evident to undergo fetal examinations (56%). The frequencies of all answers are displayed in Figure 9.

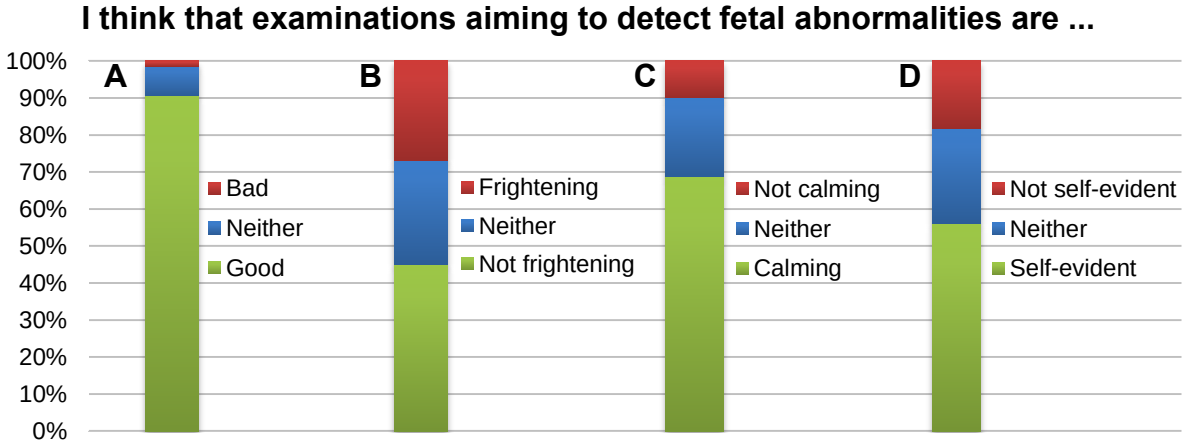


Figure 9. Pregnant women's attitudes towards prenatal examinations.

When asked about attitudes towards specific prenatal examination methods, as many 97% were positive towards ultrasound. This can probably be explained by that ultrasound is appreciated also by women who do not want to perform chromosomal screening on their fetuses, as well as that the visualization itself is an often perceived as an emotional experience that facilitates attachment[114]. Conversely, only 42% of the women were positive towards invasive testing. The positive attitudes towards NIPT and FCT were approximately similar; 73% vs. 78%, respectively.

4.7.3 Attitudes towards chromosomal abnormalities and risk perception

Almost one third of the women in the cohort (n=306, 31%) stated that it would not matter if their baby was born with a chromosomal abnormality such as Down syndrome, whereas 45% (n=450) stated that they would react negatively, and 18% (n=180) would find it very negative. The proportions did not differ between women who had previous children or not (p=0.777), or between women who had experienced miscarriages or not (p=0.382). However, women willing to use NIPT stated that they would react negatively or very negatively to a significantly higher extent than women who would not like to do the test (77% vs. 36%, p<0.001).

When the women were asked about their self-perceived probability of having a child with a chromosomal abnormality, 73% thought it was unlikely, 4% thought it was likely, and 21% stated that they did not know. The results did not differ between women below or above 35 years of age (p=0.618). The women were asked to specify what they thought was a high probability, by selecting an alternative on a scale ranging from 1:1 to 1:20,000. The most frequent answer, selected by 33% (n=224) was 1:200, *i.e.* the threshold used as high risk in FCT screening in Sweden. However, almost one third (n=301, 30%) answered "I don't know". When the women were asked to specify what they thought was their own probability of having a child with a chromosomal abnormality, as many as 58% (n=241) answered "I don't know". The answers could indicate that they either did not know, or that they did not know how to interpret the values. The challenge of communicating risk information in a comprehensible fashion has been addressed previously[115], and additionally, risk perception varies depending on factors such as personal background, gender and socio-economic status, and is highly influenced by emotions and affect[116,117]. Out of the 171 women giving an answer, 95% thought that their probability was 1:5,000 or less. This could be put in relation to the overall age associated risk, which at 32 years (*i.e.* the mean age of the participants in the study) is 1:769 for trisomy 21, and 1:323 for any chromosomal abnormality[46].

4.7.4 Decision making regarding prenatal chromosomal examinations

Almost all women (96%) stated that the decision to undergo prenatal chromosomal examinations is their own. However, the majority stated that they make the decision together with their partner (68%). Only 14% and 10% stated that they were affected by the midwife or doctor at the maternity clinic, respectively. Hopefully, this reflects that expectant parents feel

sufficiently informed to make their own choice, and that the health professionals have managed to deliver information about the examinations in an objective manner.

The main factor affecting the women's decision if to undergo fetal chromosome screening was worry about the baby's health (83%), followed by the desire to have as much information as possible about the fetus (55%). Only 3% stated that they were affected by expectations from others, and even fewer (1%) by the feeling that "everyone else is having such tests". Out of the women who did not want to use NIPT, 20% stated that nothing influenced their decision as they were already convinced that they did not want to do such tests, and another 38% did not choose any of the given alternatives, which further strengthens the hypothesis that the women who not want to perform prenatal chromosomal examinations are more convinced about their choice.

5 CONCLUDING REMARKS

The studies included in this thesis have focused on the development and evaluation of new procedures to diagnose genetic disorders in fetal life, and thereby improve prenatal care and increase our knowledge of the normal and abnormal fetal development, as well as investigation of pregnant women's attitudes towards prenatal diagnosis. The following conclusions can be drawn from the studies:

- In the analysis of stillbirth, conventional chromosome analysis is prone to failure, and QF-PCR is a useful complementary method. Analysis with CMA has a higher success rate as well as a higher aberration detection frequency than both karyotyping and QF-PCR, and therefore we conclude that CMA is a valuable tool for identification of chromosomal aberrations in stillbirth.
- Our results from analysis of 290 stillbirth cases give further support to the hypothesis that cardiac channelopathies might contribute to stillbirth. Furthermore, our results suggest an increased frequency of pathogenic SNVs in genes associated with cardiomyopathies and CHD in stillbirth compared with the general population. Screening for pathogenic SNVs in genes associated with heart disease might be valuable in cases of stillbirth where today's conventional investigation does not reveal the underlying cause of fetal demise.
- We showed that fetal tissue calcifications are highly associated with chromosomal abnormalities, especially in combination with congenital malformations. Identification of a calcification together with a malformation at autopsy more than doubles the probability of detecting a chromosomal abnormality, compared with identification of a malformation only. We propose that identification of a fetal tissue calcification at autopsy, and potentially also at ultrasound examination, should infer special attention towards co-existence of malformations, as this would be a strong indicator for a chromosomal abnormality.
- We concluded that the evaluated amplification-free sequencing protocol can potentially be used for NIPT, as we have shown that it can distinguish trisomies from healthy controls in clinical samples. Compared with other NIPT protocols, the amplification-free method can be used on smaller quantities of input DNA, reduces GC bias and increases genome coverage. The amplification-free method would be the method of choice when coverage is important, when partially degraded input material is used, or when there is a need of low amplification-bias combined with low cost and scarce material.
- Regarding attitudes towards prenatal examinations, the overwhelming majority of a cohort of 1,003 pregnant women considered analyses aiming to detect fetal abnormalities to be good. Additionally, the majority had a positive attitude towards NIPT and would like to use the test if available. The main factor affecting the women's decision to undergo prenatal chromosome examinations was worry about the baby's health.

6 FUTURE PERSPECTIVES

The results from the studies included in this thesis have provided support for the value of using various genetic analysis methods in the field of prenatal diagnosis, as well as insights to the prevalence of genetic aberrations in fetuses. In clinical practice, the results have led to fetuses with tissue calcifications being routinely analyzed by chromosome analysis. There are also ongoing discussions regarding whether to analyze all cases of stillbirth with CMA, although it has not yet been implemented.

Medical genetics is an expansive field, and the methods are rapidly shifting towards high-throughput, genome wide analyses. The development of high-resolution chromosome analysis methods, such as CMA, has without a doubt led to a massive increase in our knowledge about submicroscopic chromosomal abnormalities, and to identification of numerous syndromes. However, these techniques are already on the verge of becoming outdated, and instead replaced by sequencing technologies. Because of the increasing quality of sequencing data, together with the rapid decrease in sequencing cost, it is likely that a great proportion of genetic analyses will be substituted by whole genome sequencing (WGS) within the upcoming years.

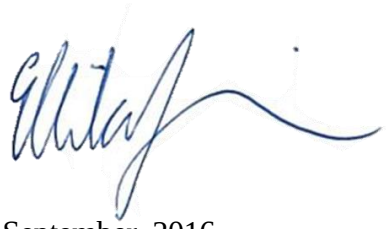
In addition to the sequence analysis in itself, WGS can successfully be used to identify CNVs[118], and has been shown to enable detection of mosaicism down to a level of ~2%[119]. However, with more data come more incidental findings, which is an even bigger issue when it comes to prenatal testing. For example, how should we handle the incidental finding of a late-onset lethal disorder? Although lethal today, there might be a treatment by the time that the fetus has become an adult person at risk of disease development. Clear and carefully prepared guidelines regarding how to handle these types of situations are needed. A way to avoid these types of dilemmas is simply to not analyze the complete genome. A good compromise could be the use of gene panels, but instead of sequencing panels, like the one used in **Paper II,** WGS is performed and an *in silico* panel is applied to the sequence data. As our knowledge about disease associated genes increases, the already available sequencing data can be reanalyzed in concerned cases. Analysis of the complete genome should only be performed in a clinical setting if there is a clear phenotype and segregation analysis is possible.

As mentioned in the "Ethical considerations" section, there are concerns regarding the "slippery slope effect" in genetic analysis, *i.e.* that the spectrum of traits tested for will widen to an extent where even non-medical indications will be included. Simultaneously, the majority of pregnant women participating in **Paper V** claimed that one of the factors affecting their decision to undergo chromosome screenings is their desire to have as much information as possible about their fetuses. The healthcare community has a responsibility to set boundaries for what is actually healthcare, to avoid shaping a society where we screen for imperfections.

Although we have come quite far in our capability to elucidate the function of protein-coding genes to interpret genetic aberrations, there is a huge gap in our knowledge when moving to oligo- or polygenic disorders. Also, we have only started to understand the regulatory components of the genome, or the functions of *e.g.* long non-coding RNA genes. In order to take the next big leap towards delineating the functions of the genome, we have to move from regarding it as a linear structure, *i.e.* solely a sequence of base pairs, to the complex three dimensional structure from which it actually performs its actions in the cell nucleus.

6.1 PERSONAL REFLECTION

When I started my studies in biomedicine near a decade ago, we were taught that 90% of the human genome consists of junk-DNA. As our knowledge deepens, this idea seems more and more naïve – why would something be junk only because we don't understand it? My personal experience has taught me that the more I learn, the more I realize how much we have yet to understand; a realization that can be frustrating, but that also makes you humble towards the intricate machinery that constitutes life, created by millions of years of evolution. Although we may never know every detail about the genome, I believe that our continuous discoveries will have a positive impact on our lives, by a better understanding of disease mechanisms and improvement of care.



September, 2016

7 SAMMANFATTNING PÅ SVENSKA

Med hjälp av fosterdiagnostik har vi möjlighet att upptäcka svåra sjukdomar redan hos foster. Att fastställa en diagnos är viktigt för omhändertagande av både fostret och de blivande föräldrarna, och vid förekomst av en genetisk sjukdom ger en fastställd diagnos dessutom möjlighet att beräkna återupprepningsrisken i eventuella efterföljande graviditeter. Kännedom om det sjukdomsorsakande anlaget har också betydelse för övrig släkt, då det kan förekomma ytterligare anlagsbärare med ökad risk att få sjuka barn. Under senare år har det skett en dramatisk utveckling av metoder som används inom det genetiska fältet. Med dessa metoder kan vi analysera stora mängder genetisk information, i en upplösning som tidigare inte varit möjlig. I detta projekt har ett flertal av dessa nya metoder använts och utvärderats, i syfte att förbättra fosterdiagnostiken och öka vår kunskap om fosters utveckling i både hälsa och sjukdom. Dessutom har vi undersökt gravida kvinnors attityder, medvetenhet och preferenser gällande fosterdiagnostik.

I **delstudie 1** studerades intrauterin fosterdöd (IUFD), vilket definieras som fosterdöd som inträffar från och med 22 fullgångna graviditetsveckor. Förekomsten av IUFD i Sverige har i princip legat konstant kring 3-4 per 1000 levande födda barn sedan 1980-talet, och trots att samtliga fall genomgår en omfattande undersökning, som innefattar fysisk bedömning/ obduktion, infektionstestning och kromosomanalys, förblir den bakomliggande orsaken till att fostren dött i många fall oförklarad. Den typ av kromosomanalys som traditionellt använts för att analysera fosterceller har använts i flera decennier, och är behäftad med vissa nackdelar. Dels har metoden en låg upplösning, vilket innebär att en eventuell kromosomavvikelse måste vara väldigt stor för att den över huvud taget ska kunna upptäckas, och dels kräver metoden cellodling, vilket är en känslig process som har en benägenhet att misslyckas – särskilt i samband med fosterdöd, då fostrets vävnader i många fall börjat upplösas, och cellerna således är i för dåligt skick för att kunna odlas över huvud taget. I fall där kromosomanalysen misslyckats har man under senare år kunnat utföra en alternativ undersökning med hjälp av en metod kallad QF-PCR *(quantitative fluorescence polymerase chain reaction).* Med denna analys kan man upptäcka aneuploidier, det vill säga förekomst av för många eller för få kromosomkopior, av ett fåtal utvalda kromosomer. Metoden har som fördel att den inte kräver cellodling, medan nackdelen är att analysen ger mycket mindre information jämfört med vanlig kromosomanalys. Under de senaste åren har nya typer av analyser utvecklats, till exempel kromosomal mikroarray (CMA). Detta är en typ av högupplöst kromosomanalys, där man genom att jämföra sitt patient-DNA med ett kontroll-DNA kan identifiera tillkomst eller bortfall av genetiskt material hos patienten. I denna studie analyserade vi 90 fall av IUFD utan en känd genetisk diagnos med CMA, för att undersöka metodens fördelar i IUFD-utredningar. Dessutom sammanställdes resultat från traditionell kromosomanalys från samtliga IUFD-fall som inträffat i Stockholms län under en femårsperiod; 2008-2012.

I sammanställningen av kliniska resultat fann vi att 7.5% av IUFD-fallen hade kromosomavvikelser som identifierats med traditionell kromosomanalys eller QF-PCR. Med

hjälp av CMA kunde vi identifiera ytterligare två kända syndrom, en avvikelse med direkt påverkan på en känd sjukdomsorsakande gen, samt 26 avvikelser av oklar klinisk signifikans. Dessutom gav CMA lyckade analysresultat av hög kvalitet i samtliga analyserade fall, till skillnad från traditionell kromosomanalys som misslyckades i var femte fall. Vi drog därför slutsatsen att CMA är ett värdefullt redskap i analysen av IUFD, då metoden ökade både andelen diagnoser och andelen lyckade analyser.

Även i **delstudie 2** inriktade vi oss på IUFD. Under senare år har rapporter publicerats som indikerar att olika typer av hjärtsjukdomar, främst störningar i hjärtats rytm, kan orsaka fosterdöd. Med hjälp av dagens sekvenseringsteknik, så kallad massiv parallellsekvensering, har vi möjlighet att sekvensera en stor mängd genetiskt material på ett sätt som fram till nyligen inte varit möjligt utan en enorm arbetsbelastning. I denna studie analyserade vi 290 fall utan en känd genetisk diagnos med en genpanel, inriktad mot 79 gener associerade med hjärtsjukdomar och hjärtats utveckling.

Vi fann att 62 (21%) av fallen hade 1-2 genetiska varianter som fanns beskrivna som sjukdomsorsakande. Majoriteten var kopplade till olika typer av rytmrubbningar, medan en andel var kopplade till påverkan på hjärtmuskulaturen eller associerade med olika former av missbildningar. Vi jämförde våra fynd med sekvenseringsdata från över 60,000 vuxna individer (tillgängligt från *The Exome Aggregation Consortium*, ExAC) och såg att de specifika genetiska varianterna var signifikant överrepresenterade bland IUFD-fallen, vilket gav ökat stöd till att de skulle kunna vara associerade med fosterdöd. Ytterligare studier är nödvändiga för att stärka sambandet mellan IUFD och hjärtsjukdom, men våra resultat indikerar att denna typ av analys skulle kunna vara värdefull i fall där den vanliga undersökningen inte identifierar den bakomliggande orsaken till fosterdöd.

I **delstudie 3** studerade vi foster med vävnadsförkalkningar. Denna typ av förkalkningar upptäcks ibland i samband med obduktion, men även, dock mer sällan, under ultraljudsundersökningar. Även om man under lång tid känt till förekomsten av förkalkningar har deras biologiska relevans inte blivit undersökt i någon större utsträckning. Spridda rapporter har indikerat en association mellan förkalkningar och infektion, cirkulationsrubbningar, missbildningar eller kromosomavvikelser. För att identifiera faktorer kopplade till förkalkningar har vi utfört en fall-kontrollstudie, innefattande 151 foster med vävnadsförkalkningar, vilket är den största grupp i sitt slag som hittills beskrivits. För varje fall identifierade vi två kontrollfoster, som var matchade mot fallen i avseende på graviditetsvecka och typ av död, det vill säga missfall, fördröjt missfall (så kallat *missed abortion*), inducerat avbrytande eller IUFD. Traditionell kromosomanalys hade tidigare utförts inom kliniken på 290 av de sammanlagt 453 fostren. De resterande 163 fallen blev analyserade med QF-PCR inom ramen för detta projekt.

Förkalkningarna var främst lokaliserade i lever, men även i hjärta, tarmar och andra vävnader. Vi fann att foster med vävnadsförkalkningar hade kromosomavvikelser i en signifikant högre grad än kontroller; 50% jämfört med 20%. De vanligast förekommande

kromosomavvikelserna inkluderade trisomi 21 (Downs syndrom), trisomi 18 (Edwards syndrom) och monosomi X (Turners syndrom). När vi jämförde fall och kontroller med kromosomavvikelser fann vi dessutom att fallen hade en signifikant högre andel missbildningar än kontrollerna. Vi fann däremot inget stöd för en association mellan förkalkningar och infektion. Vi drog således slutsatsen att förekomst av vävnadsförkalkningar hos foster är associerade med en hög risk för kromosomavvikelser, särskilt i kombination med missbildningar, och föreslår att man vid upptäckt av en förkalkning riktar särskild uppmärksamhet mot förekomst av missbildningar då det i dessa fall är hög sannolikhet att en kromosomavvikelse föreligger. Resultaten från denna studie har lett till att foster med förkalkningar numera rutinmässigt undersöks med kromosomanalys.

Att identifiera kromosomavvikelser hos foster under pågående graviditet har traditionellt gjorts med hjälp av ett invasivt test, det vill säga fostervatten- eller moderkaksprov. Dessa metoder att få tillgång till fosterceller har använts i över 40 år och är förenade med en risk för missfall i upp till 0.5% av ingreppen. Fosterdiagnostikfältet håller dock på att genomgå en omfattande förändring på grund av att icke-invasiv fosterdiagnostik, så kallad NIPT *(non-invasive prenatal testing)* håller på att etablera sig internationellt.

Under en pågående graviditet sker en stor omsättning av celler i främst placentan. Detta leder till att korta bitar av fostrets DNA läcker ut i moderns blodomlopp, och att man därmed kan få tillgång till fostrets arvsmassa genom ett enkelt blodprov. Med hjälp av massiv parallellsekvensering av detta så kallade cellfria foster-DNA (cffDNA) möjliggörs identifiering av kromosomavvikelser. Ett problem med dagens metoder för NIPT är att de innehåller ett amplifieringssteg, vilket innebär att man med hjälp av en enzymatisk reaktion mångdubblar mängden DNA som sedan ska sekvenseras. Amplifiering leder dock oundvikligen till ett systematiskt fel *(”bias”)* på grund av att vissa DNA-sekvenser amplifieras mer effektivt än andra. Om man kunde ta bort amplifieringssteget skulle säkerheten i resultaten från NIPT potentiellt kunna öka. I **delstudie 4** har vi utvärderat ett amplifieringsfritt NIPT-protokoll, i syfte att klarlägga metodens möjlighet att korrekt identifiera aneuploidier hos foster.

Metoden testades på 31 prover från gravida kvinnor, av vilka 15 bar foster med verifierade kromosomavvikelser. Samtliga 15 fall blev korrekt klassificerade som avvikande, men i ett fall där fostret bar två kromosomavvikelser; trisomi 18 och XXY, misslyckades metoden att identifiera den extra X-kromosomen. För att få klarhet i varför X-kromosomen inte identifierades gjordes uppföljande analyser med QF-PCR och CMA. Dessa visade att fostret i själva verket var mosaiker och att den extra X-kromosomen endast fanns i en andel av cellerna, medan resterande celler hade normal könskromosomuppsättning. Trisomi 18 förekom dock i samtliga celler. Denna mosaicism förklarar troligen att den extra X-kromosomen inte upptäcktes i NIPT-analysen. Vi drog slutsatsen att den amplifieringsfria NIPT-metoden potentiellt kan användas för att upptäcka kromosomavvikelser hos foster, då den visades kunna identifiera avvikelser i kliniska prover. I jämförelse med andra NIPT-

protokoll kan det användas på mindre mängder DNA, samtidigt som det minskar bias och ökar täckningsgraden.

NIPT implementerades som klinisk analys på Klinisk Genetik, Karolinska Universitetssjukhuset, i juni 2015. I samband med införandet av nya metoder är det av värde att studera hur metoden kan komma att tas emot av de potentiella användarna; i detta fall gravida kvinnor. I **delstudie 5** har 1003 gravida kvinnor rekryterats i väntrummen till nio mödravårdscentraler lokaliserade i olika delar av Stockholmsområdet, utvalda för att ge en bred representation i socioekonomisk status bland deltagarna. Kvinnorna fick fylla i en enkät i syfte att undersöka deras inställning till NIPT och huruvida de hade kännedom om metoden. Vi undersökte dessutom kvinnornas inställning till fosterdiagnostik i stort, hur deras beslut gällande att genomgå denna typ av undersökningar fattas, liksom hur information om dessa undersökningar ska ges på bästa sätt. Dessutom studerades inställningen till att få ett barn med en kromosomavvikelse, samt kvinnornas egen uppfattning om sannolikheten för att det ska ske.

Vi fann att 60% av kvinnorna hade hört talas om NIPT tidigare, men ändå uppgav 74% att de var säkra på att de skulle vilja genomgå testet om det fanns tillgängligt. Det tyder på att ett test med de egenskaper som återfinns hos NIPT är efterlängtat av den gravida populationen. Utöver ett intresse för NIPT uppgav hela 91% av kvinnorna att de var positivt inställda till fosterundersökningar över lag. Den främsta faktorn som påverkar kvinnornas beslut att genomgå kromosomundersökningar var oro för barnets hälsa (83%) följt av en önskan att vilja ha så mycket information som möjligt om fostret (55%). Majoriteten av deltagarna uppgav att de föredrar att få muntlig information om fosterundersökningar av barnmorskan på mödravårdscentralen. En tredjedel av kvinnorna uppgav att det inte hade någon betydelse ifall deras barn skulle visa sig ha en kromosomavvikelse som till exempel Downs syndrom, samtidigt som en stor majoritet uppskattade att sannolikheten att det skulle inträffa som mycket låg.

Resultaten från studierna i denna avhandling ger ökad insikt om hur olika genetiska metoder kan användas inom fosterdiagnostik. Därutöver har vi ökat vår kunskap om olika typer av genetiska avvikelser hos foster.

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